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**The RNA polymerase II C-terminal domain  
Its phosphorylation and interaction with the  
Mediator complex**

Submitted for the degree of PhD

By

Teit Max Moscote Soegaard

March 2006

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## **Abstract**

The submitted thesis describes a biochemical investigation within the field of gene transcription. In yeast (*Saccharomyces cerevisiae*) and the metazoans, the conserved RNA polymerase II (RNAPII) protein complex carries out regulated transcription. The C-terminal domain (CTD) of Rpb1 (the largest subunit of RNAPII) consists of 26 heptapeptide (YSPTSPS) repeats that are targeted by specific CTD-kinases and -phosphatases to change the phosphorylation of Ser2 and Ser5 resulting in two electrophoretically different forms of RNAPII - a fast migrating hypo-phosphorylated form and a slower migrating hyper-phosphorylated form. The changing patterns of phosphorylation modulate the interaction of multiple factors involved in transcription initiation, RNA processing, transcription elongation and RNAPII ubiquitylation throughout the transcription cycle. Prior to transcription a preinitiation complex (PIC) is assembled on the promoters of regulated genes. A plethora of regulatory pathways converge on a protein complex named Mediator - a central, functionally conserved protein complex that incorporates into the PIC to physically interact with RNAPII and which can only interact with hypo-phosphorylated RNAPII. Mediator plays the crucial role of relaying repressive and activating signals from transcription factors bound on the promoter to RNAPII poised at the beginning of the coding sequence of the gene. As Mediator is only found associated with promoter regions on chromatin RNAPII must dissociate from Mediator when transcription initiates and promoter escape occurs. The mechanism leading to dissociation of RNAPII from Mediator is unknown. A large conformational change in Mediator accompanies the association with RNAPII and the interaction is dependent both on the C-terminal domain of Rpb1 (CTD) and of other members of the RNAPII complex. If hypo-phosphorylation is needed for Mediator to interact with RNAPII does hyper-phosphorylation lead to the dissociation of the two complexes? Are other factors needed to bring about the dissociation of the holopolymerase complex? By using purified forms of Mediator and holopolymerase and purified specific CTD kinases the work presented addresses these questions in vitro.

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## Abbreviations

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### Abbreviations

ARC	Activator Recruited cofactor fraction
BRE	TFIIB Recognition Element
Cat. no.	Catalogue number
CD	Circular dichroism
CDK7	Cyclin dependent kinase 7
CDK8	Cyclin dependent kinase 8
CID	CTD Interacting Domain
CK	Casein Kinase
CRSP	Co-factor Required for Sp1 activation
CryoEM	Cry Electron Microscopy
Cs	Cold Sensitive
CTD	C-terminal heptapeptide repeat domain
CTDK-1	CTD Kinase 1
CycC	Cyclin C
CycH	Cyclin H
DNA	DeoxyRiboNucleicAcid
DPE	Downstream Promoter Element
DRB	5,6-dichloro-1-b-D-ribofuranosylbenzimidazole
DSIF	DRB sensitivity inducing factor
EC	(transcription) Elongation Complex
EDTA	EthyleneDiamineTetraAcetic
FRET	Fluorescence Resonance Energy Transfer
GTF	General Transcription Factor
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylases
holoRNAPII	Mediator-RNAPII holoenzyme
Inr	Initiator sequence
ITC	Isothermal Titration Calorimetry
MES	2-(N-Morpholino)EthaneSulfonic acid
mRNA	Messenger RNA
NAT	Negative regulator of Active Transcription
NP40	(octylphenoxy)polyethoxyethanol, NonidetP40
ORF	Open Reading Frame
PAP	Peroxidase Anti Peroxidase , soluble complex Invitrogen
PIC	PreInitiation Complex
PVDF	PolyVinylDiFlouride
RNA	RiboNucleic Acid
RNAPII	DNA-dependent RNA polymerase II
rRNA	Ribosomal RNA
RT	Room Temperature
SAGA	Spt,Ada,Gcn5
snRNA	Small Nuclear RNA
TAF	TBP associated factor
TBP	TATA Binding Protein
TCEP	Tris (2-Carboxy-Ethyl)-Phosphin-HCl
TFTC	TBP-Free TAF Complex
tRNA	Transfer RNA
Ts	Temperature Sensitive
Tween-20	Polyoxyethylene(20)sorbitan monolaurate
UTR	UnTranslated Region

## **Summary**

The largest subunit in the DNA-dependent RNA polymerase II (RNAPII) multi-protein complex, Rpb1, features a conserved C-terminal heptapeptide repeat domain (CTD) with multiple sites of potential phosphorylation. The CTD domain is well conserved through evolution, from yeast to man. Throughout the transcription cycle, dynamic changes in phosphorylation patterns occur across the CTD in active RNAPII. Specific patterns of phosphorylation correlate with phases in transcription and orchestrate CTD interactions with a host of factors involved in transcription initiation, transcription elongation and RNA processing. Only hypo-phosphorylated RNAPII is recruited to the PreInitiation Complex (PIC) and a change in phosphorylation status of the CTD from hypophosphorylated to hyperphosphorylated has been shown to coincide with the transition from transcription initiation to transcription elongation. Transcription regulation largely depends upon a multi-protein complex called Mediator, which physically relays signals from activators bound at enhancer sequences in DNA, to RNAPII in the PIC. Aside from the context of the PIC, Mediator also interacts with RNAPII in the Mediator-RNAPII holoenzyme (holoRNAPII). In the holoRNAPII complex, the interaction is mediated by a combination of the CTD and other interaction surfaces on RNAPII and the “head” domain of Mediator. Recruitment of RNAPII to the PIC has been proposed to occur either in one step in the form of holoRNAPII, or as part of a stepwise formation of the completed PIC. At the promoter-clearance stage, dissociation of the Mediator-polymerase complex takes place. In the context of the PIC, hyperphosphorylation of the CTD has been reported to promote PIC dissociation but the integrity of holoRNAPII in relation to phosphorylation has not been directly tested.

In the course of the work presented here, holopolymerase was purified by conventional chromatography and immobilised by affinity tags. CTD kinases TFIIH, TFIIF and CTDK-1 were purified and used to hyperphosphorylate the CTD.

Results show that:

1. In the context of holoRNAPII, hyperphosphorylation of the RNAPII CTD leads to dissociation of Mediator and RNAPII.
2. Dissociation can be caused by both Ser2 and Ser5 phosphorylation.
3. Mediator stimulates both TFIIF and CTDK-1 kinase activity
4. The ubiquitin ligase Rsp5 is able to stimulate CTDK-1 activity

The function of the CTD as a physical coordination platform for the mRNA production machinery makes the structure-function aspect very interesting. By NMR analysis, the CTD domain has been shown to be largely unordered. Nonetheless, changes in apparent Stokes radius upon phosphorylation have been observed, and biochemical data also point towards steric constraints on the interaction with CTD interacting proteins. Many of the proteins that interact with the CTD are also functionally interchangeable between species, implying a conservation of their mode of interaction with the CTD. Complexes of CTD interacting domains (CID) bound to CTD repeat peptides have been analysed by X-ray crystallography and specific CTD structures have been observed to be created by induced fit. A theory was proposed by which the CTD would adopt a tight structure when hypophosphorylated and an extended structure when specifically phosphorylated on Ser5 of the heptapeptide repeat. The present thesis presents an attempt to establish an experimental system to measure phosphorylation- (or factor binding-) dependent changes in the distance from end to end of the CTD using Fluorescence Resonance Energy Transfer (FRET). In the course of the presented work, CTD modifying enzymes (Fcp1, Ess1) and CTD binding factors (Rsp5, Elongator) were purified.

## **1. Introduction**

### **1.1 Brief overview of the transcription cycle**

Histone modification and chromatin remodelling of promoter regions make possible the establishment of a core of sequence-specific protein factors that nucleate the ensuing recruitment of General Transcription Factors (GTF's). Large conformational changes in DNA and the members of the PIC, promoter melting and synthesis of the first few phosphodiester bonds, as well as hyper-phosphorylation of the RNAPII CTD and transition to an active transcription Elongation Complex (EC) are all events that take place at the beginning of the so-called transcription cycle. These events have been the focus of several recent review papers (Dvir et al., 2001; Sims, III et al., 2004). The "transcription cycle" is a repeated sequence of events roughly consisting of the five sequential phases: (1) Pre-initiation complex assembly, (2) Initiation (formation of the first phosphodiester bond), (3) Promoter clearance (the point when RNAPII leaves the initiation complex and starts elongation), (4) Transcript elongation, and (5) Termination, where RNAPII is released from its template and recycled (Goodrich and

## Introduction

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Tjian, 1994). During pre- and post-initiation, the transcription complex undergoes several phases of differential stability before it commits to the task of transcription elongation (Jiang et al., 1996). The capping of nascent mRNA may constitute a further pre-promoter-clearance check-point which is possibly reflected in the dynamics of the early DNA transcription bubble (Pal and Luse, 2003) and the promoter proximal pausing of RNAPII (Pei et al., 2003). During elongation, the patterns of RNAPII CTD phosphorylation change dynamically and factors important for successful elongation (elongation factors) or co-transcriptional splicing (splicing factors) are recruited or travel along with RNAPII, often regulated by the varying patterns of CTD phosphorylation. At the end of the open reading frame (ORF), the CTD serves to coordinate cleavage and polyadenylation of the transcript and RNAPII is released from the template and dephosphorylated (recycled) before going for another round in the cycle.

The interaction between RNAPII and the Mediator complex (in the context of holoRNAPII) and the phosphorylation of the CTD of RNAPII is the major topic of my work.

In this introduction, I have chosen to describe each complex (and relevant kinases) following the train of events before, during and after transcription. The first part of the introduction will describe RNAPII and the factors needed to form a PIC and initiate basal and activated transcription. As the Mediator complex is intimately linked with this process, a more detailed description of its structure and functions will follow. Finally, events downstream of promoter clearance will be described, as well as the central role played by the RNAPII CTD in aspects of transcription, from initiation to termination. Because the work presented in this thesis was performed with yeast proteins, the main focus of the introduction is on work performed in this organism.

## 1.2 RNA polymerase II

Transcription is an ancient process carried out by proteins belonging to a conserved family of multi-subunit DNA-dependent RNA polymerases (RNAP). Although the individual members are not identical on the sequence level, functionally important regions show high homology from bacteria and Archaea to human (reviewed in (Ebright, 2000; Young, 1991)). The core of *E. coli* RNAP comprises three different



## Introduction: RNA polymerase II

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subunits known as the alpha chain, the beta chain and the beta prime chain ( $\alpha$ ,  $\beta$  and  $\beta'$ ). The complex is assembled as a hetero tetramer with the composition [ $\alpha_2$ ,  $\beta$ ,  $\beta'$ ] (Ishihama, 1981). During transcription initiation, the core polymerase is joined by the bacterial specificity factor sigma70 ( $\sigma^{70}$ ) subunit.

In contrast, the *S. cerevisiae* RNAPII is a 12-subunit complex consisting of proteins Rpb1 to Rpb12. Even so, examination of the primary sequence has found extensive homology between bacterial and eukaryotic RNAP. On the amino acid level, six short regions of Rpb1 have from 38% to 55% homology with the largest *E. coli* RNAP subunit  $\beta'$  encoded by the gene RPOC (Allison et al., 1985). Nine homology regions across Rpb2 are up to 30% identical on the amino acid level with *E. coli* subunit  $\beta$ , the second largest RNAP subunit encoded by the RPOB gene (Sweetser et al., 1987). Furthermore, Rpb3 and Rpb11 have homology to the *E. coli* RNAP sigma factor (Young, 1991). The yeast Rpb6 subunit was shown to be homologous structurally and functionally with the non-essential bacterial RNAP omega factor ( $\omega$ ) (Minakhin et al., 2001). Thus the Rpb1, -2, -3, -6, and 11 are designated the “conserved subunits” of RNAP (Ebright, 2000).

Additionally, Rpb4 has amino acid sequence homology to  $\sigma^{70}$ , which in bacteria enables the core RNAP to bind specifically to start site regions, initiate transcription efficiently, and respond to regulatory signals. Like  $\sigma^{70}$ , the Rpb4/Rpb7 dimer is a dissociable sub-complex of eukaryotic RNAPII (Orlicky et al., 2001).

### ***1.2.1 Three types of RNAP in eukaryotes***

In eukaryotes, a division of labour exist amongst three different types (type I, II & III) of nuclear DNA-dependent RNA polymerases. RNAPI is active in nuclear superstructures called nucleoli, where it transcribes the ribosomal DNA genes to create ribosomal RNA (rRNA), the precursor to the nucleic acid portion of the large ribosomal structures. RNAPII is responsible for transcription of all protein-encoding (mRNA) genes, as well as small nuclear RNA (snRNA), while RNAPIII handles the synthesis of transfer RNA (tRNA) and 5S rRNA (Young, 1991) and some other snRNP's. Five subunits of RNAP in yeast (ABC27/Rpb5, ABC23/Rpb6, ABC14.5/Rpb8, ABC10- $\beta$ /Rpb10, and ABC10- $\alpha$ /Rpb12) are shared between the three types of RNAPII and are designated “the common” subunits (Carles et al., 1991).

The homology between the metazoan RNAPII genes allowed a probe derived from the sequence of the RPII215 gene (encoding the largest subunit of *Drosophila melanogaster* (*D. melanogaster*) RNAPII) to be used to isolate and clone the *Saccharomyces cerevisiae* (*S. cerevisiae*) genes RP021 and RPO31, encoding the largest subunits of RNAPII and RNAPIII (Rpb1 and Rpc1 respectively) (Allison et al., 1985; Biggs et al., 1985). Whereas the overall homology on the amino acid level is 35%, Rpb1 and Rpc1 are highly homologous in six sequential regions. In these regions, the homology is 43-71% on the amino acid level and between 43% and 74% on the nucleotide sequence level. The general level of homology indicates that the RPII215, RPO21 and RPO31 genes may have arisen through a process of gene duplication and divergence. The main difference between Rpb1 and Rpc1 lies in a C-terminal heptapeptide repeat domain, which is found only in Rpb1 (Allison et al., 1985). Functionally, six mammalian RNAPII subunits can substitute for their yeast RNAPII counterparts (Rpb6, -7, -8, -9, -11 & -12) in plasmid shuffling experiments (McKune et al., 1995; McKune and Woychik, 1994), a clear functional reflection of the very homologous sequences, and underscoring the concept of RNAPII as an evolutionarily fundamental mRNA-producing protein machine.

### **1.2.2 Overall architecture of Yeast RNAPII**

Temperature sensitive mutations that hinder the assembly pathway of *S. cerevisiae* RNAPII were identified in genes coding for Rpb1, -2 and -3. By co-immunoprecipitation of combinations of epitope tagged proteins synthesised at permissive or restrictive temperature Rpb1 was shown to interact with a Rpb2/Rpb3 complex (Kolodziej and Young, 1991). Further, in *Schizosaccharomyces pombe* (*S. pombe*) far-Western and chemical crosslinking studies of the pair wise interaction of recombinant RNAPII subunits demonstrated that Rpb1 and Rpb2 form a core around which the rest of the RNAP subunits assemble. Additionally, Rpb3 forms a substructure with Rpb5 and -11 (Ishiguro et al., 1998).

### **1.2.3 The dissociable Rpb4/Rpb7 sub- complex**

Yeast RNAPII was originally purified as a 12 subunit complex (Edwards et al., 1990; Kolodziej et al., 1990), but crystallisation attempts failed because of the tendency of RNAPII preparations to be non-homogeneous due to the presence or absence of the

dissociable Rpb4/Rpb7 sub-complex (Darst et al., 1991). Around 80% of RNAPII complexes purified from yeast cells in log phase growth are devoid of the Rpb4/Rpb7 sub-complex. Only when purified from cells in early stationary phase does the 12-subunit complex become stoichiometric (Choder and Young, 1993). While dispensable for non-specific transcription initiation in a purified system *in vitro*, addition of purified Rpb4/Rpb7 to the reaction restores the ability of RNAPII to specifically initiate transcription (Edwards et al., 1991).

### ***1.2.4 Flexible domains within RNAPII***

Several conformational changes within the RNAPII complex accompany its interaction with DNA. A domain designated “the clamp” is composed of regions of Rpb1, -2 and -6 and is connected to the main complex by mobile “switch regions”. As a part of one side of the “cleft” in RNAPII that accommodates the DNA template, the clamp is able to change position in a up to 30 Å transition from “open” to “closed” conformation (Cramer et al., 2000). Closing is thought to be induced by DNA-protein contacts within the cleft and also by the binding of Rpb4/Rpb7 on the surface of RNAPII. The sub-complex wedges into a groove at the base of the clamp structure, which restricts the mobility of the clamp, locking it into position, hence causing the polymerase to close around the DNA strand (Armache et al., 2003). The first crystal structure of 12 subunit RNAPII (PDB ID code: 1NIK) indeed showed the clamp in the closed confirmation (Bushnell and Kornberg, 2003), whereas two previous structures (PDB ID code 1i3Q & 1i5O) of RNAPII devoid of Rpb4/Rpb7 showed the clamp in open confirmation (Cramer et al., 2000; Cramer et al., 2001). As only 12-subunit RNAPII is competent for transcription initiation, the role for Rpb4/Rpb7 as a locking “wedge” creates the problem of how the DNA helix reaches the active site in RNAPII (Asturias and Craighead, 2003). Entry of DNA into the RNAPII groove would either entail dissociation and re-association of Rpb4/Rpb7 as RNAPII engages the template, or the closed conformation would serve to ensure that only melted promoter DNA can reach the active site at the transition from promoter-closed to promoter-open conformation (Armache et al., 2003). In contrast to the X-ray analysis, cryoEM analysis of the 12-subunit RNAPII at 18 Å resolution showed a much more mobile clamp configuration. In images where RNAPII orientation allowed the clamp position to be discerned (80% of total), only 25% were in the clamp closed position and 75% were

in the open conformation. Furthermore, analysis of a RNAPII  $\Delta$ Rpb4/Rpb7 complex showed that 40% of RNAPII were in clamp-closed conformation, suggesting that Rpb4/Rpb7 is dispensable for clamp closure per se (Craighead et al., 2002). The position of Rpb7 is close to where the CTD linker exits from the main RNAPII structure, which may also be where the nascent RNA strand exits (Bushnell and Kornberg, 2003; Gnatt et al., 2001). Indeed, recombinant Rpb7 has been shown to bind both DNA and RNA in band-shift assays, pointing to a post-initiation role in the transcription cycle (Orlicky et al., 2001).

### **1.3 C-terminal heptapeptide repeat domain**

As mentioned above, a domain of heptapeptide repeats situated at the C-terminus of Rpb1 sets the largest subunit of RNAPII apart from RNAPI and RNAPII. The consensus repeat sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser is well conserved from yeast to metazoans (reviewed in (Corden, 1990)) and serves as a platform for coordination and assembly of proteins involved in nearly all aspects of transcription (reviewed (Hirose and Manley, 2000)). In this thesis, I will suffix each amino acid with its position in the repeat.

#### ***1.3.1 The overall structure of the CTD***

A 100 amino acid domain (from residue 1436 to residue 1535) of Rpb1 is known as “the linker” as it links the CTD domain to the remainder of the protein (Cramer et al., 2001). Theoretically, if these domains formed a rigid, extended structure, the linker would have a length of 280Å and the CTD would be 650Å long (Cramer et al., 2001), thus potentially exposing a very large surface which could be used for interaction with proteins involved in transcription. Both the linker and CTD fail to generate diffraction patterns when RNAPII crystals are analyzed using X-rays, which clearly indicates that both are inherently unordered, very flexible domains. The absence of “ordered-ness” of the CTD initially obscured the site of its attachment to the remaining non-CTD RNAPII complex. The site was eventually identified by replacing 5 residues (from Pro1430 to Gly1434) with an HA epitope followed by two recognition sites for factor Xa protease enabling the generation of RNAPIIB completely devoid of the CTD domain (Li and Kornberg, 1994). Electron crystallography of 2D crystals of RNAPII located the position of the 12CA5 antibody (bound to the HA epitope) in the structure, and crystals of RNAPII with and without the CTD were furthermore compared to identify electron



density resulting from the CTD. By lowering the detection threshold, an elongated sphere of electron density was revealed close to the binding site of 12CA5 at the C-terminus of Rpb1. The absence of a corresponding density in RNAPIIB crystals indicated that it indeed arose from the CTD of RNAPIIA. The region measured approximately 100 Å across and its volume was roughly four times the calculated theoretical volume of the CTD, indicating a high degree of flexibility of the CTD (Meredith et al., 1996). Whether the shape and size of the CTD electron density is merely a consequence of restraints on the mobility of CTD that confines it to the space available in the crystal, or whether this indeed is the way the CTD is organized *in vivo* is debatable. The co-planar RNAPII structure occupying the same unit cell did not have a comparable CTD electron density, indicating a different degree of freedom of movement at other sites in the crystal lattice, which argues that the structure of the crystal itself indeed significantly influenced the apparent shape of the CTD (Meredith et al., 1996).

### ***1.3.2 Phosphorylation states of the CTD***

Three isoforms of the largest subunit of *D. melanogaster* RNAPII designated -IIA, -IIB and -IIO were originally identified by their differential mobility in SDS-PAGE. The fastest migrating band (RNAPIIB) was shown *in vitro* to arise from proteolytic cleavage of purified RNAPIIA, whereas the slowest moving isoform (RNAPIIO) could be created by phosphorylation of RNAPIIA (but not RNAPIIB) with casein kinase type I (CK1). This implied that what is cleaved off RNAPIIA to create RNAPIIB is the CTD, and also that this is where the phosphorylation sites reside (Cadena and Dahmus, 1987; Greenleaf et al., 1976). The basis for the phosphorylation dependent mobility shift of Rpb1 has been studied by several groups. Incorporated radioactivity (using  $\gamma^{32}$  labelled ATP) was compared to the length of mobility shift of purified calf thymus RNAPII and demonstrated that a mobility shift of 80% of the full distance could be caused by phosphorylation at a level of only 30% of the highest level of radioactivity measured (Payne and Dahmus, 1993; Payne et al., 1989). A phosphorylation level corresponding to approximately 40% of the highest level was sufficient to shift a CTD peptide band quantitatively in SDS-PAGE (Zhang and Corden, 1991b). Hence, RNAPIIA to IIO mobility shift in and of itself is not proof of phosphorylation at every potential phosphorylation site. A time-course analysis of the phosphorylation reaction using

Urea-PAGE gels rather than SDS-PAGE gels showed a gradual faster migration of the CTD peptide from the hypo- to the hyper-phosphorylated state correlating with increasing negative charge due to the phosphorylation (Zhang and Corden, 1991b). The interpretation was that due to increased electrostatic repulsion, initially the negative charge from the phosphate groups will reduce the affinity of SDS molecules for the CTD causing a slower mobility of the hyperphosphorylated CTD.

### ***1.3.3 Processive or distributive mechanism of phosphorylation***

Hyper-phosphorylation of RNAPII by the TFIIH associated kinase, under non-limiting conditions, does not appear to create forms of intermediate mobility in SDS-PAGE, but rather results in a quantitative shift of the whole band to the higher position in the gel. If the system is not limiting in substrate, and the reaction has gone to completion, this is indicative of a processive mechanism where the kinase phosphorylates several potential sites, before it disengages its substrate (Feaver et al., 1991; Morris et al., 1997). Unlike TFIIH kinase, the purified CTD kinase Srb10-Srb11 is found to engender intermediate SDS-PAGE mobility shifts of GST-CTD fusion proteins *in vitro* (Borggreffe et al., 2002). This is indicative of a distributive mechanism of action, where only very few phosphates are incorporated in the CTD for each kinase-substrate encounter (Pinhero et al., 2004).

## **1.4 A model for CTD structure**

The mobility shift of the hyperphosphorylated CTD in SDS-PAGE may be explained by the repulsion of strongly negatively charged SDS molecules from the increasingly negatively hyperphosphorylated CTD, leading to a less negative charge overall and hence slower migration in the gel. Nonetheless, observations of the CTD in solution make a case for the existence of some sort of secondary structure or tendency for “ordered-ness” in the CTD.

### ***1.4.1 Changes in apparent CTD size dependent on phosphorylation state***

A mouse CTD peptide was phosphorylated using either E2 kinase or Casein Kinase II (CKII) giving rise to the IIO and IIA form, respectively. E2 kinase phosphorylates maximally 15-20 repeats out of 52 while CKII has a single recognition site at the extreme C-terminus of the CTD (Dahmus, 1981; Zhang and Corden, 1991a) Sedimentation analysis by ultra centrifugation through a 5-18% sucrose gradient

revealed a Svedberg sedimentation value of 2.9 for IIO and 2.2 for IIA. Thus, by sedimentation analysis, the IIO form is much heavier than the IIA form. The difference is greater than would be expected based from the mass difference assuming the incorporation of 15-20 phosphates in the IIO from of the CTD peptide, which lead the authors to propose that hyperphosphorylation causes the CTD peptide to assume an extended shape (Zhang and Corden, 1991b). Interestingly, by further Superose 6 gel-filtration analysis, the authors also observed a shift in stokes radius upon phosphorylation. The CTD IIA peptide with a theoretical Mw of 40 kDa had an apparent Mw of 65 kDa, suggesting a slightly extended shape. Upon hyperphosphorylation, the peptide now eluted close to a Mw of 130 kDa, pointing to a phosphorylation-dependent change in the structural dynamics of the CTD peptide, going from a partially extended to a fully extended state in RNAPIIO in support of the results found by sedimentation analysis (Zhang and Corden, 1991b).

Other indications for the existence of a CTD secondary structure comes from the differential preference of CTD kinases for substrate length. Recombinant purified human cyclin H/CDK7/p36Cdk8 (TFIIK in yeast) or human cyclin C/CDK8 (formerly Srb11/Srb10 in yeast) was used to phosphorylate GST-CTD fusion proteins containing 4, 6, 8 or 10 consensus repeats. A clear preference of cyclin H/CDK7/p36Cdk8 for the longer sequences was observed. Short sequences in turn, were preferred by cyclin C/CDK8 (Rickert et al., 1999). As the substrate sequences were identical, only differing in their number of repeats, this may indicate the existence of secondary CTD structures that the two kinases recognise differently. Recent data indicate that the mammalian CTD carries a three-repeat region at the base of the CTD that interacts with an essential acidic 10 amino acid sequence at the CTD-end, to stabilise Rpb1 against enzymatic degradation (Chapman et al., 2005). The molecular mechanics underlying this observation are unknown.

### ***1.4.2 NMR analysis of CTD peptides***

#### **1.4.3 CTD structure attained by induced fit**

The lack of a solid structure of the CTD domain has been documented in numerous papers based on its absence in RNAPII crystal structures (Cramer et al., 2001) and on NMR analysis of both a few CTD repeats (Cagas and Corden, 1995; Harding, 1992; Kumaki et al., 2001; Noble et al., 2005) as well as of the full-length recombinant murine CTD domain (Bienkiewicz et al., 2000). The solution structure of CTD peptides

## Introduction: A model for CTD structure

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has also been studied extensively by circular dichroism (CD) analysis (Bienkiewicz et al., 2000; Cagas and Corden, 1995; Nishi et al., 1995).

The overall result of these analyses seems to be that the CTD contains less than 20%  $\beta$ -turn structure in H<sub>2</sub>O. This  $\beta$ -turn proportion increases to 90% in Trifluoroethanol, which favours hydrogen bonds. The  $\beta$ -turns occur around the two SPXX motifs in the YSPTSPS sequence. One report found the  $\beta$ -turn around the SPTS motif to be slightly more stable than the SPSY motif (Kumaki et al., 2001).

On the whole, these investigations found little evidence of inherent stable conformations within the CTD peptide. Rather, the CTD has been suggested to adopt different conformations by a mechanism of induced fit, as cognate factors bind to it. In the crystal structure of the WW domain of the cis-trans isomerase Pin1 (homologous to yeast Ess1) bound to a CTD peptide phosphorylated at Ser2 and Ser5, the CTD peptide was shown to assume an extended coil structure and both the two Ser-Pro bonds were in the *trans*-conformation (Verdecia et al., 2000). A different structure was suggested by the crystal structure of the RNA guanylyl-transferase Cgt1 bound to four CTD repeats phosphorylated at Ser5. In this case each CTD repeat was in a non-extended  $\beta$ -like conformation (Fabrega et al., 2003).

The crystal structure of the CTD interaction domain of a conserved and essential subunit of the yeast cleavage and polyadenylation factor Pcf11, bound to a Ser2 phosphorylated CTD peptide, indicated that the CTD peptide had a tendency to form a  $\beta$ -fold around Ser2-Pro3-Thr4-Ser5. The  $\beta$ -fold formed independently of interactions with its binding partner and so was probably not created by induced fit (Meinhart and Cramer, 2004). Extrapolation from this observation prompted the formulation of a model, according to which a hypo-phosphorylated CTD would form a compact left-handed spiral consisting of  $\beta$ -turns with 1.8 CTD repeats per winding. The compacted spiral would be 100 Å long in accordance with from the crystal structure described by (Meredith et al., 1996), rather than the predicted length of 650 Å for a fully extended CTD. Hypothetically, Ser5P hyperphosphorylation of the CTD would cause the spiral to extend (due to static repulsion between phosphate groups), whereas Ser2P would be sterically compatible with the spiral structure because the Ser2P would face outward of the spiral, and furthermore, Ser2P was postulated to stabilise the  $\beta$ -fold structure (Meinhart and Cramer, 2004). A different study of the crystal structure of the Pcf11 CID and a Ser2 phosphorylated three-repeat CTD peptide, suggested that a non-extended

conformation was assumed by the peptide upon binding, but this study found no evidence for a Ser2P induced stabilisation of the  $\beta$ -fold (Noble et al., 2005).

Taken together the case is strong for a role of induced fit in CTD conformation. Thus, the overall structure of the CTD may well dramatically change upon modification, or binding of factors. If, indeed, the CTD assumes a more extended conformation in the hyper-phosphorylated state (Meinhart and Cramer, 2004), a possible consequence would be an increase in protease sensitivity caused by an increase in exposure to solvent. Interestingly, and in agreement with this notion, RNAPIIO purified from elongating mammalian transcription complexes is much more sensitive to proteases than is the RNAPIIA form purified from the soluble fraction (Laybourn and Dahmus, 1989).

The differences described above, between the largely unordered CTD structure found by NMR analysis and the indications for an extended CTD structure, measured by gel-filtration and gradient centrifugation are interesting. In the second part of the Results section of this thesis, the development of a system to measure potential expansion of the CTD domain is described. This system will be used to measure the effect of CTD phosphorylation and/or binding of CTD interacting proteins.

## 1.5 CTD genetics

### 1.5.1 Co-evolution of CTD and the transcription apparatus

Phylogenetic analysis of CTD sequences identified a subset of eukaryotes composed of animals, plants, fungi, and several related groups of uni-cellular organisms that all show consensus for only the first six CTD positions of the heptad repeat consensus. The interpretation was that these species shared a common ancestor and therefore made up a so-called “clade” (defined as the CTD-clade) (Stiller and Hall, 2002). Variation especially in the last residue (Ser7), but also in Ser2 and Thr4, create non-consensus repeats that normally occur most frequently in the C-terminal half of the CTD domain in metazoans. Both the length of the repeat domain and the number of non-consensus repeats within a CTD vary between species; thus the number of non-consensus repeats is 21 (of total 52) in human and 19 (of total 26) in yeast (Corden, 1990), whereas the more divergent *D. melanogaster* CTD features a mere two heptads out of 44 repeats that exactly match the consensus sequence (Barron-Casella and Corden, 1992; Stiller et al., 2000; Stiller and Hall, 2002). Even more extreme, *Plasmodium falciparum* RNAPII carries only a single consensus repeat out of 17 (Li et al., 1989; Prelich, 2002).

The evolutionary constraints imposed on the CTD sequence were proposed to be a result of the interactions of the host of proteins that participate in the production of mRNA (Corden and Patturajan, 1997), or in the proposed transcription factories in the nucleus (Iborra et al., 1996). As a platform for protein-protein interaction, the CTD enables complex interaction mechanisms of multiple factors during mRNA synthesis and thus within the CTD-clade the requirement for consensus repeats was suggested to be tightly linked with the emergence in evolution of a more complex transcription machinery (Stiller and Hall, 2002). Support for this notion recently came through phylogenetic sequence analysis of the CTD-clade, which suggested parallel evolution of the primary sequence of the CTD and of proteins interacting with it (Guo and Stiller, 2005).

### ***1.5.2 The diheptad repeat is the functional unit***

Recent data suggest that the functional unit of the CTD sequence lies in diheptad repeats rather than single repeats. The plasmid shuffle method (Sikorski and Boeke, 1991) was used to force yeast cells to survive on a mutant RPO21 gene encoding different versions of the CTD, and viability was measured. While the insertion of a single alanine residue between every individual repeat is lethal, insertions of single alanines between pairs of repeats in tandem, is almost without growth defect compared to wildtype sequence (Stiller and Cook, 2004). The insertion of two alanine residues does give an intermediate growth defect, but up to 5 alanine residues can be inserted while still retaining yeast viability. Interestingly, support for the importance of tandem repeats also comes from recent Isothermal Titration Calorimetry (ITC) analysis of the interaction between the CTD Interaction Domain (CID) of the yeast mRNA 3'-end processing factor Pcf11 and CTD heptad peptides. The data suggested that one repeat was insufficient for binding and that the binding site overlapped two repeats. Furthermore, the CID domain can change rapidly between two binding sites, suggesting that multiple adjacent sites will significantly increase the affinity of the CID domain for the CTD (Noble et al., 2005). These observations have support in much earlier functional studies on TFIIF kinase activity. When purified human TFIIF (Conaway et al., 1992) was used to phosphorylate a tri-repeat peptide, the Ser5 position in repeats 2 and 3 were phosphorylated 13 times more efficiently than the same position in the first repeat

(Trigon et al., 1998) possibly implying that TFIIF prefers to bind one repeat while phosphorylation the adjacent repeat.

### ***1.5.3 CTD truncation suppressor genes***

Progressive shortening of the CTD showed that in *S. cerevisiae*, a length of at least 8 repeats is essential for survival and between 9 and 12 repeats still causes Temperature Sensitive (tc) and Cold temperature Sensitive (cs) phenotypes, as well as inositol auxotrophy and slow growth. Wild type growth requires an RNAPII with more than 12 consensus repeats (Nonet et al., 1987; West and Corden, 1995). A genetic screen for *S. cerevisiae* genes that suppress the cold sensitive phenotype of a truncated CTD domain lead to identification of the *Srb* genes (Suppressor of RNA polymerase B mutation) *Srb2*, -4, -5, -6, and -7. Subsequently, they were cloned and recombinantly expressed and polyclonal antibodies were generated (Nonet and Young, 1989). These proteins, which are subunits of the Mediator complex, will be discussed in further detail below.

### ***1.5.4 Effects of CTD truncation in mammalian cells***

The effect of CTD truncation in mouse cells was tested using stable transfection of cells in culture with plasmids expressing an  $\alpha$ -amanitin resistant mutant version of the largest mouse RNAPII subunit (encoded by the *RPII215* gene). The presence of  $\alpha$ -amanitin inhibits the endogenous RNAPII, forcing cells to survive on the recombinant mutant ( $\alpha$ -amanitin resistant) polymerase. Non-lethal mutations in the CTD domain of the  $\alpha$ -amanitin resistant polymerase would thus confer  $\alpha$ -amanitin resistance to the cells. This system enables the *in vivo* study of different RNAPII mutations and a version of it was first described by (Bartolomei et al., 1988).

Results indicated that mouse cells minimally need around 30 CTD repeats for viability, while cells with 32 -36 CTD repeats exhibit a slow growth phenotype. Repeats with divergent sequence are less important for viability than consensus repeats, and deletion of 21 C-terminal repeats (encompassing all the non-consensus repeats from the 52 repeat sequence) has no severe phenotype (Bartolomei et al., 1988). The apparent dispensability of conserved, non-consensus CTD repeats in mammalian cell culture prompted an investigation of the importance of non-consensus repeats in mice. A knock-out mouse was created that had a homozygous deletion of 13 repeats (9 of which were non-canonical) of the *Rpb1* CTD. The resulting mouse was smaller than wild type

littermates and had a higher rate of neonatal lethality, suggesting a role for the full-length CTD in regulating growth during development (Litingtung et al., 1999).

### ***1.5.5 CTD substitution mutations***

Mutation of Serine to Alanine is commonly used to ascertain the functional effect of absence of a phosphorylation site. Mutation of Serine to Glutamine is supposed to mimic a constitutively phosphorylated Ser position, because of the negatively charged Glutamate side chain. Mutation in all CTD repeats of Ser2 or Ser5 to alanine or glutamine is lethal in yeast (West and Corden, 1995). Interestingly however, the N and C-terminal regions of the CTD have different tolerance for substitution mutations. Variant non-consensus repeats are most abundant in the C-terminal part of the CTD and include Ser2 to T substitutions, Thr4 to S/Q, and Ser7 to E/G/K/N/R/T/V (Corden et al., 1985). The plasmid shuffle system was used to show that in a strain that retained a total of 7 wt repeats, a Ser5→Glu substitution in five repeats in the N-terminal half of the CTD was lethal, while the same substitution in up to seven repeats was viable when situated in the C-terminal half of the CTD. Conversely, Ser2→Glu substitution of seven repeats in the N-terminal half was viable, but moving the sequence of substituted repeats to the C-terminal proved lethal (West and Corden, 1995).

Interestingly, in this respect, CTD kinases also differ in their ability to phosphorylate either N- or C-terminal halves of the CTD. CycH/CDK7/MAT1 and CycC/CDK8 were expressed and purified in the baculovirus system and used in an investigation into their substrate preference. The substrate was GST fused to the full-length, the N-terminal or the C-terminal part of the mouse CTD. In this system, phosphorylation of a peptide with four repeats containing the Ser7→K substitution was more efficient with CycH/CDK7/p36Cdk8 than with cyclin C/CDK8, indicating a preference of CycC/CDK8 for the consensus repeats in the N-terminal part of the CTD sequence (Rickert et al., 1999). Using a GST-CTD fusion protein, CTDK-1 kinase was reported to be highly selective towards consensus repeats compared to repeats with the Ser7→L substitution (Morris et al., 1997) suggesting that this kinase may also prefer to phosphorylate the N-terminal part of the CTD.

Together, these results indicate that both the number and position of different phosphorylation sites are important for CTD function. *In vivo*, functional differences



between the C- and the N-terminal parts of the CTD sequence may mirror the differences in repeat phosphorylation.

In the mouse CTD, the C-terminal portion (including heptads 27–52) was found to support capping, splicing, and 3' processing, whereas the amino terminus portion only supported capping (Fong and Bentley, 2001). Indeed, a 10 amino acid sequence at the extreme C-terminal of Rpb1 was shown to be required for mRNA synthesis, polyadenylation and capping (Fong et al., 2003). The molecular basis for this is yet unknown.

Having described the main structural features of RNAPII, I now turn to the functional aspects of RNAPII life. In the following paragraphs, I will describe particular events that occur during the process of gene transcription.

### **1.6 Gene activation**

Nucleosomes snugly cover genomic DNA, organising it into dense super-structures (Luger and Hansen, 2005). Multiple events have to be coordinated in order to activate a gene, not least because chromatin structures present a strong barrier to efficient transcription. *In vitro*, these nucleosomal obstacles severely impede transcription, while *in vivo*, transcription is enabled due to factors that de-condense the chromatin structure in various ways (Svejstrup, 2004). Some, but not all, activator proteins can recognise and bind enhancer sequences in chromatin, and it is generally assumed that their main role is to recruit chromatin remodelling complexes as well as parts of the general transcription machinery to the promoter regions of genes (Orphanides and Reinberg, 2002).

#### ***1.6.1 Chromatin remodelling complexes***

The family of ATP dependent chromatin remodelling complexes in yeast comprise Swi/Snf, RSC, Ino80, SWR-C, Chd1, and ISWI (ISW1a, ISW1b, and ISW2) (reviewed in (Becker and Horz, 2002; Narlikar et al., 2002). These complexes work by changing the distribution of nucleosomes in chromatin, thus uncovering regulatory DNA sequences. Additionally, histone acetyl transferases (HAT's) and histone deacetylases (HDAC's) cooperate to regulate the level of acetylation of specific lysine residues in the N-termini of histones (Roth et al., 2001). Originally thought to function merely by neutralising positive charges on the lysine residues, to loosen chromatin structure

around the negatively charged DNA fibre (Allfrey et al., 1964), they are now recognised to be part of a larger group of enzymes, which modify specific positions in histone N-terminal tails by methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation to create what some have termed a “histone code” (Jenuwein and Allis, 2001). These marks are then recognised by the chromatin remodelling complexes (which move or displace nucleosomes), or indeed by other regulatory proteins. The HAT containing SAGA complex and its relations to the GTF TFIID are described below.

### 1.6.2 TFIID and SAGA

Many promoters contain a variable TATA box sequence, which is recognised by a complex of TATA binding protein (TBP) and TBP associated factors (TAFs). Recombinant TBP from *D. melanogaster* was found to support accurate initiation of basal, - but not activated transcription *in vitro* (Hoey et al., 1990). At the same time partially purified TBP from *D. melanogaster* cells did support activated transcription *in vitro*, suggesting that something was missing from the recombinant TBP (Pugh and Tjian, 1990). The advent of TBP antibodies made possible the isolation of a complex that included TBP and TAF's (Dynlacht et al., 1991) and could substitute for the crude TBP containing fraction to support activated transcription *in vitro*. The GTF TFIID is required for transcription from all three types of promoter and is functionally conserved from yeast to man (Kelleher, III et al., 1992).

Yeast TFIID is a multi-protein complex, consisting of TBP and up to 14 other TAF's (Sanders and Weil, 2000) and TFIID components also contribute to other complexes. For instance, the yeast nuclear HAT Gcn5 was shown to associate with a complex termed SAGA (Spt,Ada,Gcn5) (Grant et al., 1997). The SAGA complex has since been purified in different versions, but the largest SAGA complex (1.8 MDa) contains Spt3, Spt7, Spt8, ,Spt20/Ada5 and Ada2 Tra1, Ada1, Med16 (Sin4), and the TFIID components TAF5(TAFII90), TAF6(TAFII60), TAF9(TAFII17/20), TAF10(TAFII25/23), TAF12(TAFII61/68) (Grant et al., 1998b; Grant et al., 1998a) (original nomenclature in parenthesis (Tora, 2002)).

TBP on its own, is unable to bind the TATA sequence when it is obscured by a nucleosome and therefore nucleosomes covering the TATA sequence must be displaced or moved by ATP-dependent chromatin remodelling complexes as a prelude to PIC

assembly (Imbalzano et al., 1994). As SAGA arrives at a promoter in chromatin, its Gcn5 subunit acetylates the histones covering the sequence, probably making the DNA more accessible to other factors and indeed purified HAT's, (SAGA, Ada, NuA4, or NuA3) were reported to stimulate transcription from a nucleosomal template *in vitro* (Steger et al., 1998).

### ***1.6.3 Transcription independent of TBP***

The TBP-Free TAF Complex (TFTC) was isolated from HeLa nuclear extract through affinity purification on single stranded DNA and affinity purification of the TFIID component hTAF<sub>II</sub>30 (Wieczorek et al., 1998). Repeated depletion of TBP (by affinity chromatography) from the elution fraction of the anti-hTAF<sub>II</sub>30 column left proteins in the flow-through which co-migrated in gel-filtration analysis, suggesting a complex of TAF's. Using a heat treated nuclear extract from HeLa cells, the TFTC complex was shown to be able to nucleate a preinitiation complex and support GAL-VP16 activated transcription from the AdMLP promoter as efficiently as TBP-containing TFIID.

### ***1.6.4 Stepwise formation of the preinitiation complex***

The TBP subunit of TFIID binds specifically to TATAAA sequences (TATA boxes) found -30 bp to -25 bp upstream of the transcription start site in many, but not all, eukaryotic RNA polymerase II promoters. In lieu of a TATA box, a second motif, the Initiator sequence (Inr) is a feature of many metazoan promoters (though the two also occur in combination), and whereas recombinant TBP alone cannot recognise TATA-less promoters, the TFIID complex includes TAF's that recognise the Inr motif, leading to recruitment of TFIID and the remaining GTF's. Specifically the TAF<sub>II</sub>250 and TAF<sub>II</sub>150 subunits of TFIID have been implicated in Inr recognition (Smale and Baltimore, 1989; Verrijzer et al., 1995). In budding yeast, transcription begins 40 -120 bp downstream of the TATA box and a Inr-like sequence found near the transcription start site probably serves as a (non-essential) positioning sequence. Generally, the Inr element is less important in yeast than in metazoans (reviewed (Smale and Kadonaga, 2003)). Other sequence elements of the core metazoan promoter that are recognised by specific proteins include the TFIIB recognition element (BRE) and the downstream promoter element (DPE) (Kadonaga, 2002; Smale and Kadonaga, 2003).

The order of recruitment of the rest of the factors needed for activated transcription is a matter of variability. The isolation of a large Srb-TBP complex (the RNAPII holoenzyme) which supported activated transcription in the presence of TBP and contained RNAPII and Srb-Mediator subunits as well as TFIIB, TFIIH and TFIIF lead to the suggestion that PIC assembly occurs in one single step following the recruitment of TFIID (Koleske and Young, 1994; Liao et al., 1995).

A parallel model has the PIC assembly occurring in a stepwise fashion, in a conserved process that has been studied in systems from yeast to human. According to this view, the bend in DNA that results from the binding of TBP (as part of TFIID) to the TATA box (Nikolov et al., 1995) creates a DNA-protein structure that is recognised by TFIIB (Forget et al., 1997). TFIIB recognises and binds the BRE sequence which lies just upstream of the TATA box, which confers directionality to the PIC assembly on human TATA containing promoters (Tsai and Sigler, 2000). Mutants in the SUA7 gene encoding a subunit of TFIIB lead to a change of initiation site to a position further downstream, demonstrating a role for TFIIB in transcription start site selection (Pinto et al., 1994). This was further confirmed by swapping TFIIB and RNAPII from *S. pombe* into an *S. cerevisiae* transcription system *in vitro*, which gave rise to transcriptional initiation from the start site characteristic of *S. pombe* (Li et al., 1994).

TFIIA recruitment (which can take place at any step after TFIID recruitment) serves to stabilise the interactions between TFIID, TFIIB and DNA (Orphanides et al., 1996; Ranish et al., 1999) and photo-crosslinking experiments indicate that human TFIIA and TFIIB each interact with 2.5 turns of the DNA helix and that this interaction with DNA is what drives the incorporation of the two into the PIC (Lagrange et al., 1996). Other experiments suggest that the assembly of TFIID, TFIIB & TFIIA (the TFIID-IIA-IIB complex) may create a nucleosome-like structure that might wrap DNA upstream of the transcription start site around it (Hoffmann et al., 1996; Oelgeschlager et al., 1996; Robert et al., 1998).

Stable recruitment of RNAPII to the PIC depends on the presence of TFIIF and happens, at least partly, via an interaction of the N-terminus of TFIIB with TFIIF. This recruits TFIIF and RNAPII to the promoter forming the DABpolF complex (Flores et al., 1991). Affinity chromatography purification of epitope tagged TFIIF from the soluble fraction of yeast extract isolates a stoichiometric complex of TFIIF and 12 subunit RNAPII (including the Rpb4/Rpb7 sub complex) (Krogan et al., 2002). CryoEM analysis of TFIIF/RNAPII shows interaction of TFIIF with Rpb1 as well as

with the Rpb4/Rpb7 sub complex of RNAPII (Chung et al., 2003). The presence of the dissociable Rpb4/Rpb7 complex is not a prerequisite for recruitment of RNAPII *in vitro* (Orlicky et al., 2001), but the absence of Rpb4/Rpb7 sub-complex renders RNAPII incompetent for activated transcription in yeast nuclear extracts (Edwards et al., 1991). Recruitment of RNAPII stabilised through TFIIF could be a way to ensure that only initiation-competent RNAPII is used to build the PIC.

After formation of the DABpolF complex, the recruitment of TFIIE follows. TFIIE interactions in the PIC were studied extensively using human proteins. Immobilised recombinant human TFIIE has been shown to selectively co-IP human RNAPIIA from a mixture of purified RNAPIIA and –IIO. Additionally, interaction of the largest subunit of TFIIE with Rpb9 probably leads to TFIIE recruitment to the DABpolF complex (Van Mullem et al., 2002). TFIIE was also shown to bind TFIIF, TBP and TFIIH by co-IP using immobilised TFIIE (Maxon et al., 1994).

TFIIE stimulates TFIIH-activities in two ways. Firstly, TFIIE recruits TFIIH and stimulates the TFIIH CTD kinase (Kin28) four-fold (Serizawa et al., 1994). Secondly, the small subunit of TFIIE was shown to stimulate the helicase activity of XBP (Ssl2 in yeast) in holoTFIIH. The effects of mutations in TFIIE indicated that this activity was essential for transcription initiation from a linear template, fitting nicely with a role for TFIIE in stimulating promoter melting (Lin and Gralla, 2005). Interestingly, by ChIP, the genome wide localisation of RNAPII in budding yeast cells was studied over a 9 day period as the culture progressed into- and out of- stationary phase. Results indicated that rather than RNAPII being recruited to genes at the beginning of a new growth phase, RNAPIIA resided on DNA in hundreds of upstream inter-genic regions, poised for fast activation at the proper signals (Radonjic et al., 2005). In fact, it was suggested that the inactive transcription machinery in stationary phase cells was “parked” in chromatin in this manner. The finding indicates that recruitment of RNAPII is not always the limiting step in gene activation.

### 1.7 Transcription initiation and the CTD

Early experiments pointed to a role for the CTD in regulation of activated transcription based on correlation between progressive truncation of the CTD and lack of transcriptional response to activators in nuclear extract (Liao et al., 1991).

Evidence for the existence of factors that regulate transcription via the CTD came from *in vitro* transcription assays in crude or defined transcription systems. Thus,

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when RNAPIIA or RNAPIIB were added to a heat treated nuclear extract from a strain expressing a *ts* allele of Rpb1, promoter specific transcription was dependent on the presence of the CTD but in contrast, in a fully defined transcription system, RNAPIIA and RNAPIIB showed equal activity in non-specific and promoter specific (basal) transcription indicating that the CTD is dispensable for RNAPII basal transcription (Li and Kornberg, 1994).

On the other hand, a role for CTD phosphorylation was suggested by the observation that in yeast extract, basal transcription by RNAPII was abolished by addition of the CTD kinase inhibitor H-8 (N-[2- (methylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride) while it was unaffected in a minimal, fully defined transcription system with purified GTF's from yeast (Li and Kornberg, 1994) or rat (Serizawa et al., 1993) indicating that in this system, transcription does not depend on CTD phosphorylation. The interpretation was that in the crude extract, due to the presence of the CTD, RNAPIIA was able to overcome repressive effects of inhibitory factors present in the extract, whereas RNAPIIB could not. Back-addition to the assay of specific Biorex70 ion exchange chromatography fractions both demonstrated the repressing activity as well as the CTD-specific “de-repressing” activity, substantiating the hypothesis (Li and Kornberg, 1994).

### ***1.7.1 Possible functions of the CTD in PIC assembly***

By immunoblot of PIC's assembled on an immobilised template in yeast extracts from cells expressing a RNAPII with only 9 CTD repeats, the amount of Mediator and RNAPII subunits as well as TFIIB on the promoter, was observed to be reduced 10 fold compared to wildtype (Ranish et al., 1999). More recently, effects of CTD truncation on PIC formation was investigated in HeLa cells by mutating the CTD of a  $\alpha$ -amanitin resistant RNAPII. An enhanced green fluorescent protein (EGFP)-tagged version of RNAPII was analysed *in vivo* using fluorescence recovery after photobleaching (FRAP) to compare the truncated CTD to wildtype. Results indicated that RNAPII with a truncated CTD was significantly more mobile (close to free diffusion) than the populations of wildtype RNAPII engaged in elongation or initiation, underscoring the importance of the length of the CTD for stable integration into initiation- and/or elongation complexes (Lux et al., 2005). Together, these observations suggest a requirement for the RNAPII CTD for efficient recruitment of Mediator and TFIIB to the

PIC, and also stress the importance of the intact CTD, for proper RNAPII association with the PIC but the CTD may serve other coordinating tasks as well.

By photo-crosslinking experiments, the CTD has been shown to contact DNA from 16 to 26 bp downstream of the transcription initiation site (Douziech et al., 1999), and a role of the CTD as a DNA intercalating agent has also been proposed (Huang et al., 1994). Furthermore, the CTD has been shown to interact directly with TBP *in vitro* (Koleske et al., 1992; Usheva et al., 1992; Usheva and Shenk, 1994), and TBP was shown to bind a single unphosphorylated heptapeptide immobilised on a resin (Usheva et al., 1992), suggesting that recruitment of RNAPII to the PIC could be mediated or stabilised in part, by direct CTD interaction with DNA and/or PIC components.

Early on, RNAPII entry into the PIC was reported to require it to be in the hypophosphorylated state. This preferential recruitment of RNAPIIA was demonstrated by a competition experiment between human RNAPIIA and RNAPIIO for entry into PIC's assembled onto a adenovirus-Major Late promoter (Ad2-MLP) template (described in (Weil et al., 1979)) in "reconstituted" HeLa cell extract (Chesnut et al., 1992). The preference for recruitment of hypophosphorylated RNAPII to the PIC is independent of the presence of the Mediator complex, as even PIC's assembled *in vitro* using homogeneously purified GTF's have been reported to prefer the RNAPIIA form (Lu et al., 1991; Zawel et al., 1995). Therefore, phosphorylation may in some way affect either RNAPII conformation and/or its interaction with DNA or GTF's in the PIC.

The monoclonal antibody 8WG16 raised against the hypo-phosphorylated form of the CTD was observed to inhibit activated transcription *in vitro* when added to a defined transcription system, purified from rat liver. Inhibition took place at a stage before PIC assembly and was manifested only when purified TFIID was used in the reaction and was absent when recombinant TBP was used (Conaway et al., 1992). Furthermore, in a similar system RNAPIIO could in fact initiate transcription, but only if yeast yTFIID was used as the TBP source, and not if the endogenous rat TBP factor was used (Serizawa et al., 1993). On the other hand, the extent of phosphorylation state of the CTD is hard to determine precisely as the gel shift in SDS-PAGE does not depend on exhaustive phosphorylation of all sites (see later) and immunoblotting may pick up signal from one phosphorylated repeat out of many unphosphorylated ones.

The dependence on the presence of TBP-associated factors for recruitment to the PIC of RNAPIIO may support a model where the CTD mediates RNAPII recruitment to the PIC via TFIID.

### ***1.7.2 Abortive transcription***

The “closed promoter” complex designates the point where a PIC is fully assembled and the promoter DNA helix is as yet un-melted. Three steps lead from the promoter-closed stage to productive transcription: strand opening, bubble movement, and promoter escape (Holstege et al., 1997). The initial phase is hallmarked by RNAPII undertaking abortive, non-productive transcription, primarily creating di- or tri-nucleotide oligomers which continually dissociate from the template, as the short DNA:RNA hybrid is very unstable and easily melts (Luse and Jacob, 1987).

Though not essential for synthesis of the first phosphodiester bond, the TFIIF complex, which melts the promoter, does stimulate the synthesis of di-nucleotides five-fold in the abortive transcription assay and is essential for promoter clearance from relaxed templates *in vitro* (Kumar et al., 1998). Inhibition of the TFIIF helicase activity by ATP- $\gamma$ S at this stage causes the melted DNA to collapse (Holstege et al., 1997). After more than 2 nucleotides are synthesised the risk of abortion is reduced somewhat, presumably because the DNA:RNA hybrid is stable enough (in the context of RNAPII) not to spontaneously melt (Gnatt et al., 2001). After the stage of 4 nucleotides, the process is no longer sensitive to inhibition of the TFIIF helicase and transcripts are no longer generally aborted, indicating a shift toward more productive transcription.

Recently, the co-crystal of RNAPII and the GTF TFIIB revealed that the so-called “Finger” domain of TFIIB contacts an area near the active site of RNAPII called “the Saddle” going via the Cleft in RNAPII (Bushnell et al., 2004). By surface plasmon resonance using an immobilised DNA template strand as ligand and RNAPII, short RNA’s and TFIIB as analytes, the TFIIB-dependent stabilisation of a ternary complex containing five-nucleotide RNA was demonstrated. *In silico* modelling showed that although TFIIB initially stabilises the short RNA:DNA hybrid strand, more than a nine nucleotide RNA strand will lead to a competition between RNA and TFIIB for occupation of the Saddle area. The differential outcome of this competition could possibly provide an explanation for the phenomenon of abortive transcription (Boeger et al., 2005). Nevertheless, aborted transcripts of up to 15 nucleotides in length can be isolated *in vitro* (Holstege et al., 1997), indicating that the transcription complex has not yet fully reached productive elongation phase at position +15.



The TFIIH complex is essential for both transcription and nucleotide excision repair (NER) and is conserved from yeast to man (Feaver et al., 1993; Svejstrup et al., 1995; Wang et al., 1995). As TFIIH is central to events during transcription initiation, including promoter melting, CTD phosphorylation and promoter escape (reviewed in (Svejstrup et al., 1996b)), a closer look at this complex is presented in the following.

### **1.7.3 The TFIIH complex**

The coreTFIIH complex comprises the ATP-dependent, 5'→3' helicase Rad3 (XPD in humans) (Sung et al., 1987) and the subunits Tfb1, -2, -4 and Ssl1 (or p62, p55 and p34 and p44, respectively, in humans). A recently identified 8 kDa protein, Tfb5, was found to be a stoichiometric complex member, raising the number of coreTFIIH subunits to 6 (Giglia-Mari et al., 2004; Ranish et al., 2004). Associated with coreTFIIH is a three-member sub module, in yeast called TFIIK, consisting of a cyclin-dependent kinase Kin28 (MO15/CDK7 in human), its cyclin Ccl1 (Cyclin H in human) and the subunit Tfb3 (or Mat1 in human) (Feaver et al., 1994; Svejstrup et al., 1995; Takagi et al., 2003). The ATP-dependent, 3'→5' helicase Ssl2/Rad25 (XPB in humans) joins the coreTFIIH complex and TFIIK, to form holoTFIIH (Svejstrup et al., 1994).

HoloTFIIH is essential for activated transcription while NER activity depends on the association of coreTFIIH and Ssl2 with the products of a number of genes involved in NER (Rad1 (human XPF), Rad2 (human XPG), Rad4 (human XPC), Rad10 (human ERCC1) and Rad14 (human XPA) to create a suggested “repairosome”. TFIIK is not required for NER (Svejstrup et al., 1995). The recruitment of a stable repairosome has been challenged by *in vivo* data from cultured human cells with defects in components of the NER repair subunits. Adherent cultured human cells can be irradiated with UV-light shone through a polycarbonate filter with 3 µm pores to cause localised damage to genomic DNA in speckles (Volker et al., 2001). This technique was used combined with immuno-cytochemistry to measure the recruitment of components of the NER pathway to the sites of damage. Cells were irradiated and fixed in intervals, and the data indicated that the recruitment of NER factors was sequential, rather than simultaneous in a repairosome.

Given the essential role of TFIIH in DNA repair along with its evolutionary conservation, it is not surprising that mutation in the human homologous complex is found to be the cause of DNA repair disorders. The human hereditary DNA repair

disorders Xeroderma pigmentosum (XP) and Trichothiodystrophy (TTD) are caused by mutation in genes encoding human TFIIH subunits. Specifically, mutations in XPB and XPD (encoding helicases) cause XP, while mutation in human Tfb5 correlates with TTD-A (Giglia-Mari et al., 2004; Ranish et al., 2004).

#### ***1.7.4 TFIIH and open complex formation***

As described above, holoTFIIH contains three subunits with ATPase activity: two ATP dependent helicases (Ssl2 (XPB) and Rad3 (XPD)) and one CTD kinase (Kin28 (CDK7)). Only the helicase activity of Ssl2 is required for transcription, as a mutation of the Walker motif in this protein is lethal in yeast, whereas it only gives NER defects when introduced in the Rad3 protein (Sung et al., 1996).

The function of the helicase activity in transcription initiation has been the subject of quite a number of publications. Early studies revealed that for transcription of topologically relaxed, linear templates in a defined transcription system, TFIIH and TFIIE were required for transcription initiation, while both factors become dispensable for transcription from negatively supercoiled templates. It was further shown that TFIIH helicase activity is indeed what becomes superfluous on supercoiled templates (Parvin and Sharp, 1993; Timmers, 1994). A defined system containing RNAPII and GTF's was used to investigate the relative importance of TFIIH helicases in promoter escape. Recombinant TFIIH complexes devoid of TFIK were assembled using helicase subunits mutated in their ATPase motif. These complexes were then tested by transcription assays using a derivative of the adenovirus major late (AdML) promoter which contained a pre-melted region from positions 29 to 21 relative to the normal transcriptional start site, essentially obviating the requirement for TFIIH mediated promoter melting. The results revealed a need for the Ssl2 helicase (but not the Rad3 helicase) for elongation up to 18 nucleotides (Moreland et al., 1999), implying a post-initiation role of Ssl2, separate from the ATP-dependent helicase activity thought to melt the promoter at initiation (Jiang and Gralla, 1995).

Later work showed, that on the artificial pre-melted AdMLP (-9/+1) promoter, in a defined transcription system, impediment of promoter clearance by TFIIF could be relieved by TFIIH (Yan et al., 1999). Based on photo-crosslinking of a TFIIF heterotetramer to DNA regions both up and downstream of RNAPII (Robert et al., 1998) it was suggested that the necessity for TFIIH helicase activity for promoter clearance

came from the need to break TFIIF-DNA interactions in order for transcription to proceed (Yan et al., 1999).

TFIIH-mediated promoter melting creates a bubble in DNA that presumably both serves to expose template bases and enables the DNA to bend so that the template strand bases can reach the active site in RNAPII (Cramer et al., 2000), leading the way for initiation of synthesis of the nascent mRNA chain. Melted regions of the non-template strand in the AdML promoter can be mapped by exploiting the sensitivity of thymidine residues in single-stranded DNA to potassium permanganate (KMnO<sub>4</sub>) (Nielsen, 1990). Using this method, a requirement for the full D/B/Pol/F/E/H complex and ATP for promoter melting was observed (Holstege and Timmers, 1997). Furthermore, formation of an open complex was found to happen in two steps: preceding synthesis of the first phosphodiester bond, ATP-dependent melting of the promoter upstream of the transcription start site from position -9 to +1 occurs. In a second step, formation of the first phosphodiester bond drives expansion of the melted region to position +8.

The exact point in the transcription process where the transcription Elongation Complex (EC) can be considered to have escaped the promoter region has been the subject of some debate. One point was suggested by recent KMnO<sub>4</sub> analysis of early transcription complexes. Synthesis of the 7<sup>th</sup> RNA phosphodiester bond results in sudden collapse of the melted region (or bubble) from -9 to +8 relative to the transcription start site, suggesting that the early transcription complex undergoes a major structural transition which may constitute a promoter clearance point because bubble collapse also suppresses the tendency of RNAPII to stall near the promoter (Pal et al., 2005).

KMnO<sub>4</sub> foot-printing of promoter regions *in vivo* has also been used to study the role of TFIIH. Whereas the *GAL1* and *GAL10* promoters had a changed pattern of KMnO<sub>4</sub> reactivity in a *ssl2-ts* strain, melting of the *TDH2* promoter was abolished suggesting a promoter-specific dependence on Ssl2 for promoter melting (Ostapenko and Gileadi, 2000). Recently, the protein-protein interactions within the PIC have been studied by DNA-protein photo-crosslinking and based on these results, *in silico* modelling created a model of the structure of the PIC (Chen and Hahn, 2004). The XBP subunit of TFIIH was shown to interact with DNA downstream of RNAPII (Douziech et al., 1999). Yet other photo-crosslinking studies of TFIIH stimulated promoter melting using purified human TFIIH *in vitro* showed that TFIIH-DNA contacts were downstream from the melted region rather than in it. The authors propose that the XPB

(Ssl2) helicase activity is used to rotate the DNA in front of RNAPII. As the DNA is fixed by the PIC, this rotation causes tension that melts the pairing of bases in the DNA helix between the PIC and ERCC3 (Ssl2) (Kim et al., 2000).

### ***1.7.5 Promoter clearance and CTD phosphorylation***

Hyperphosphorylation of the RNAPII CTD precedes promoter clearance, and in addition, some observations suggest that RNAPII is rapidly dephosphorylated after leaving the promoter. In a crude, reconstituted transcription system from HeLa cells (that depended on the addition of exogenous RNAPII for transcription) photo-crosslinking using a special UTP analogue (4-thiouracil) showed that only RNAPIIO cross-linked to mRNA transcripts of maximally 15 nucleotides (nt) length from the Ad2-MLP promoter (Payne et al., 1989). When the system was supplied with RNAPIIA, still only RNAPIIO was cross-linked to RNA, implying that hyperphosphorylation takes place before promoter clearance. In a similar system, RNAPIIA was observed to be preferentially cross-linked to longer (27 – 40 nucleotides) RNA while RNAPIIO was cross-linked to nucleotides of 16-23 nucleotide length, possibly indicating that in a crude system the CTD is accessible to CTD specific phosphatases only once RNAPII has left the PIC (Bartholomew et al., 1986).

The conversion of RNAPIIA to RNAPIIO, coincident with promoter clearance, suggested that CTD hyperphosphorylation could be a necessary step of transcription initiation. This was challenged by *in vitro* work using purified GTF's on a Ad2MLP template while specifically inhibiting kinase activity in the reaction, which showed that RNAPII was actually able to clear the promoter independent of RNAPII phosphorylation (Serizawa et al., 1993). Again, the phosphorylation state of RNAPII was only probed by immunoblotting of the repeated CTD epitope, opening the possibility, that regions of the CTD may have been hypo-phosphorylated in these experiments.

ATP analogs such as Adenylylimidodiphosphate (AMP-PNP) or adenosine-5'-[ $\gamma$ -thio] triphosphate (ATP $\gamma$ S) in which the  $\gamma$ -phosphate group is not (or only slowly) hydrolysable, can be used to inhibit the ATP-dependent helicases in TFIIH. Using this approach, TFIIH helicase activity was suggested to be necessary to keep open the bubble in promoter DNA as its inhibition by incubation with ATP $\gamma$ S resulted in bubble collapse (Dvir et al., 1996). The TFIIH helicase activity was suggested to be necessary

to bring about a conformational change in the components of the PIC to allow RNAPII to clear the promoter region and leave the scaffold (described below) behind (Forget et al., 2004) and the energy for the PIC conformational change has recently been suggested to be stored in the tension of the growing initial transcription bubble and to be released at the point of bubble collapse (Pal et al., 2005).

The length of the nascent RNA was reported to affect the stability of the EC. Cutting the nascent transcript shorter than 50 nucleotides by exonuclease treatment destabilises the EC indicating that RNA has some role to play in the remarkable stability of elongating RNAPII (Ujvari et al., 2002), and possibly offers supporting evidence for the torpedo model of transcription termination, which will be presented in slightly more detail below (Buratowski, 2005).

### ***1.7.6 A protein scaffold stays behind***

After the promoter clearance step, RNAPII leaves the PIC to travel across the open reading frame (ORF) of the gene. This event is coupled to disassembly of parts of the PIC and *de novo* assembly of an EC, nucleated by elongating RNAPII. The disassembly process has been documented in various ways. In a purified system, a template competition assay was performed in which the migration of human GTF's and RNAPII from one template to another was measured by transcriptional readout (Zawel et al., 1995). It was shown that while TFIID remains bound to the promoter after initiation, TFIIB dissociates from the PIC at the point of initiation, but is to some extent later re-recruited to the scaffold. The time of dissociation of TFIIB fits well with the point of RNA competition for occupation of the space near the saddle structure in RNAPII (Boeger et al., 2005). TFIIIE dissociates from RNAPII during synthesis of the first 10 nucleotides whereas TFIIF dissociates from RNAPII sometime after this point, but before 30 bases have been synthesized. The last factor to leave the elongation complex (EC) is TFIIH, which dissociates between 10 and 68 bases downstream of the initiation site. If RNAPII is stalled due to nucleotide starvation, TFIIF is able to re-associate and upon restart of RNAPII, it leaves the EC again (Zawel et al., 1995). By *in vitro* transcription in mammalian extracts, it was confirmed that TFIIH is not a part of the elongation complex after the synthesis of 30 nucleotides (Ping and Rana, 1999).

The existence on initiated promoters of a GTF-containing “scaffold” that stimulates re-initiation was recapitulated in yeast nuclear extracts by assembly of PIC's

on immobilised templates. Transcription was initiated using a recombinant Gal4-AH or Gal4-VP16 activator and factors that remained bound to the promoter after initiation were identified by immunoblots (Yudkovsky et al., 2000). While TFIIB, TFIIIE and RNAPII leave the promoter at initiation, the activator, the Mediator complex, TFIID, TFIIA, TFIIH and TFIIE remain and form a scaffold structure that stimulates re-recruitment of TFIIB, TFIIF and RNAPII for subsequent rounds of transcription.

The Mediator complex makes up the major part of the PIC and it is needed to support activated transcription from the promoters of most regulated genes. The following is a closer look at this important complex.

### 1.8 Isolation of Mediator

The first example of activated transcription in a fully defined system was demonstrated in the lab of Roger Kornberg in 1994 (Kim et al., 1994). Whereas basal transcription from a minimal promoter comprising a TATA box and transcription start site can be reconstituted from purified RNAPII and five general transcription factors (GTF): TBP, -IIB, -IIF, -IIE, and -IIH, transcription stimulated by activator proteins (Gal4-GCN4 or Gal4-VP16) had previously only been seen in systems where a crude (or less well defined) component was added. An activity that could mediate a transcription activation signal from the activator to the basal transcription machinery was evidently missing from the fully defined system. During purification of yeast GTF's, a fraction that could mediate activated transcription in such a system was derived from the development of a hydroxyapatite column (Kim et al., 1994). The mediating activity co-purified over several chromatographic steps and stemmed from a complex apparently consisting of tens of proteins besides RNAPII and TFIIF. This complex was named holo-RNA polymerase II (holoRNAPII), and it did not contain TFIIB, TFIIH or TBP as did a previously reported holoenzyme (Thompson et al., 1993). However, when combined with purified GTF's [TBP, TFIIB, -IIE, -IIF] and activators (GCN4 or GAL4-VP16) the complex did support activated transcription from a minimal promoter template (Kim et al., 1994). The large complex behaved on gel-filtration as a 1.9 MDa complex. TFIIF was initially thought to be associated with the Kornberg Mediator, but later experiments showed that only Mediator, and not TFIIF, was displaced from holoRNAPII by incubation with anti-CTD 8WG16-affinity resin (Svejstrup et al., 1997), indicating that Mediator, but not TFIIF, binds directly to the CTD. Indeed,

refinements in the purification procedure (Li et al., 1996) also lead to the isolation of Mediator devoid of TFIIF. Based on the transcription activation assay, the Mediator complex was purified to virtual homogeneity as a ~21 subunit complex with a molecular mass of ~1 MDa that interacts directly with the CTD of RNAPII (Bushnell et al., 2004; Kim et al., 1994).

### ***1.8.1 Mediator and activated transcription***

Budding yeast whole genome microarray analysis of ts mutants of Mediator subunits Med17 (Srb4), Med18 (Srb5) and Med6 shows that a vast majority of genes are down-regulated more than 2-fold at the restrictive temperature (Holstege et al., 1998). Initially, Mediator was thought essential for all activated transcription, but dispensable for transcription of housekeeping genes (Gerber et al., 1995). Furthermore, genome-wide mRNA microarray analysis of  $\Delta med2$  and  $\Delta med16$  (*sin4*) strains under galactose or heat-shock growth conditions demonstrated a >2-fold down-regulation of two, largely separate groups of around 200 highly expressed genes. Other highly expressed genes were unaffected by the mutation, indicating a gene-specific role for Mediator subunits for activated- but not for basal transcription (Myers et al., 1999).

### ***1.8.2 Mediator orthologs***

Based on sequence homology to *S. cerevisiae* Mediator genes for Srb4, Med7 and Nut2, three open reading frames from the *S. pombe* genome were cloned, expressed, purified and used for antibody production. Affinity purification using these antibodies as well as 8WG16 (against the RNAPIIA CTD) lead to the isolation of a *S. pombe* holoenzyme containing RNAPII and spSrb4, spMed7 and spNut2 proteins (Spahr et al., 2000). Later investigations revealed that *S. pombe* Mediator is much smaller than the *S. cerevisiae* Mediator (390 kDa compared to 1012kDa) and comprises 13 subunits, all but three of which, have homologs in other eukaryotes (Spahr et al., 2001) indicating a high degree of conservation, despite the difference in size. The *S. pombe* Mediator exists in two forms, one that is devoid of the [spSrb8/ spTRAP240/ spSrb10/ spSrb11] sub-complex which is homologous to the *S. cerevisiae* [Med12/ Med13/ Cdk8/ CycC] complex. Functional conservation of Mediator complexes in yeast is indicated by the ability of the *S. pombe* Mediator devoid of the [spSrb8/ spTRAP240/ spSrb10/ spSrb11] complex to

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stimulate activated basal transcription *in vitro*, while the spMediator variant including the complex had repressive function on basal transcription *in vitro* (Spahr et al., 2003).

Orthologous Mediator complexes have also been identified in metazoans. Initial sequence comparisons failed to show any significant homology of yeast Mediator proteins to proteins in other species, indicating that Mediator might be restricted to yeast. However, the isolation from human HeLa cells of a protein complex, Thyroid hormone Receptor associated Protein (TRAP), which, like Mediator, enabled activated transcription (Fondell et al., 1996) as well as the isolation of the vitamin D Receptor Interacting Proteins (or DRIPs) that could support transcription activated by the Vitamin D<sub>3</sub> Receptor (VDR) in a Namalwa B-cell cell extract, suggested that protein complexes of similar function to Mediator were conserved through evolution (Rachez et al., 1998). Also, the resemblance in overall structure of the complexes seen by cryo-EM analysis (Dotson et al., 2000) prompted further scrutiny of the sequences. More recently, Multidimensional Protein Identification Technology (MudPIT) was used to confirm the existence of conserved mammalian Mediator-RNAPII proteins (Sato et al., 2004). Indeed, using bioinformatic search techniques, the human Mediator complexes have since been established as a true orthologs of yeast Mediator (reviewed in (Boube et al., 2002; Conaway et al., 2005)).

One orthologue, the human Activator Recruited Cofactor fraction (ARC) consists of two distinct complexes, ARC-L and a co-factor required for Sp1 activation (CRSP)(Ryu et al., 1999; Ryu and Tjian, 1999). Like Mediator, the CRSP complex mediates activator-dependent transcription *in vitro* and includes conserved orthologs of yeast Mediator middle and head domains proteins Med1, Med7, Med14 (Rgr1), & Med17 (Srb4) (Bourbon et al., 2004). In contrast, ARC-L contains a Cdk8 (Srb10) homologue and is inactive in stimulation of activated transcription (Taatjes et al., 2002). Another proposed human Mediator ortholog is the negative regulator of activated transcription (NAT) (Sun et al., 1998). This ~20 subunit complex was isolated by chromatographic fractionation (Phosphocellulose and DEAE) of a HeLa nuclear extract and immunoprecipitation using an antibody against Cdk8. Besides Cdk8 (hSrb10) and CycC (hSrb11), the complex was reported to contain the Mediator orthologs hMed6, Rgr1 (Med14) and Srb7 (Med21). Interestingly, NAT was able to repress activated transcription in a defined system *in vitro*, when using either RNAPIIA and RNAPIIB but not when adding RNAPIIO, suggesting that the hyperphosphorylation of the CTD might be obstructive to interaction with NAT (Sun et al., 1998). However, the large



number of non-Mediator subunits in NAT begs the question whether NAT really is a true Mediator ortholog. A orthologous Mediator complex has also been isolated from *D. melanogaster* (Park et al., 2001). In summary, the Mediator complex is conserved from yeast to man, but occurs in a wealth of different compositions across species.

### ***1.8.3 Mediator architecture from Head to Tail***

Mediator architecture was first teased out using stepwise dissociation of stable sub-complexes by treatment of immobilised holoRNAPII with a gradient of Urea. This identified two Mediator sub-modules, one containing Med14 (Rgr1) and another containing Med6/Med17 (Srb4) (Lee and Kim, 1998). In order to refine the architectural Mediator map, co-IP of pairs of recombinant epitope-tagged Mediator subunits (expressed in the baculovirus system) as well as further stepwise dissociation studies on immobilised purified Mediator were undertaken. Results showed the Med17 (Srb4) sub-complex to consist of [Med20 (Srb2), Med17 (Srb4), Med18 (Srb5), Med22 (Srb6), Med6, Med8, Med11, Med19 (Rox3)] and also delineated a new sub-complex: the Med9/Med10 complex containing [Med1, Med4, Med7, Med9, Med10, Med21 (Srb7)] (Kang et al., 2001). The three domains of Mediator (Head, Middle and Tail) were defined based on extensive pair-wise interaction studies of recombinant Mediator subunits, as well as cryoEM analysis of the purified complex. According to this model, the Med17 (Srb4) sub complex corresponds to the head domain while the Med9/Med10 containing complex corresponds to the Middle domain. The tail domain comprises [Med2, Med3, Med14(Rgr1), Med15(Gal11), Med16(Sin4)] (Dotson et al., 2000; Guglielmi et al., 2004). Finally, the Cdk8 domain containing [Med12, -13, Cdk8, CycC] associates with a subset of Mediator complexes through interaction of Med13 (Srb9) with the head domain subunit Med17 (Srb4) (Guglielmi et al., 2004).

### **1.8.4 The [Med12, -13, Cdk8, CycC] sub-module.**

The presence or absence of the [Med12, -13, Cdk8, CycC] sub-module in Mediator preparations has been fiercely debated.

While the Young lab found the kinase-cyclin pair Cdk8 (Srb10/CycC (Srb11) to be a stoichiometric part of the RNA polymerase II holoenzyme (Liao et al., 1995), the Kornberg lab purified a Mediator of transcriptional activation completely devoid of Cdk8 (Srb10) (Kim et al., 1994). Investigating this discrepancy, the Young lab repeated the purification using the Kornberg procedure while employing an activated

## Introduction: Isolation of Mediator

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transcription assay to follow Mediator activity and using antibodies raised against recombinant Med12, -13 & Cdk8. The result was co-elution of all nine Srb proteins (including Med12, -13 & Cdk8), leaving the matter unresolved (Hengartner et al., 1995). Later findings indicate that some preparations of holoRNAPII contain trace amounts of the [Med12 (Srb8), Med13 (Srb9), Cdk8 (Ser10), CycC (Srb11)] complex, which is more abundant in holoRNAPII prepared from cells in log phase of growth (Hengartner et al., 1998; Holstege et al., 1998).

Immunoprecipitation from crude yeast nuclear extracts using tagged Mediator subunits under gentle, low salt conditions (250mM KAc) resulted in the isolation of a large complex comprising both the [Med12 (Srb8), Med13 (Srb9), Cdk8 (Ser10), CycC (Srb11)] subunits and RNAPII (a holoRNAPII complex) as well as the previously identified Mediator subunits and a smaller (550 kDa) Mediator complex comprising [Med1-9, Med10(Nut2), Med11, Med17(Srb4), Med18(Srb5), Med20(Srb2), Med21(Srb7) and Med22 (Srb6)], but devoid of RNAPII and [Med12 (Srb8), Med13 (Srb9), Cdk8 (Ser10), CycC (Srb11)]. This complex was named Mediator core (Medc) (Liu et al., 2001).

In a parallel effort to characterise the composition of human Mediator complexes, a crude HeLa nuclear extract was subjected to gel filtration chromatography under gentle conditions (0.3 M KCl) in order not to disrupt native protein complexes. Immunoblotting demonstrated a major ~ 2 MDa multi-protein complex which included CDK8/Cyclin C (yeast Srb11), as well as Sur2 (Med23), hMed6 and DRIP150 (Med14/Rgr1), and a minor ~150 kDa complex containing only CDK8/CyclinC. The large complex was devoid of RNAPII, but stimulated activated transcription in a defined system, whereas the smaller complex was a transcriptional repressor (Wang et al., 2001).

### 1.9 RNAPII-Mediator complexes

Immunodetection of Srb proteins through several chromatographic steps enabled the purification of a large 1.2 MDa multi protein complex designated the Srb-TBP complex (Thompson et al., 1993). Later, the complex was termed the RNA polymerase II holoenzyme and, aside from the Srb proteins, it contained RNAPII, and the basic factors TFIIB, TFIIF & TFIIF. The complex contained only traces of TBP, but adding recombinant TBP enabled it to respond to activation by GAL4-VP16 activator in promoter driven transcription *in vitro* (Koleske and Young, 1994). More work

## Introduction: RNAPII-Mediator complexes

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established that [Med20(Srb2), Med17(Srb4), Med22(Srb6), Med18(Srb5), Med21(Srb7), Med12(Srb8), Med13(Srb9), Cdk8(Srb10), CycC(Srb11)] were part of the large complex (Hengartner et al., 1995). The complex had a combined theoretical mass of 1.4 MDa and an apparent molecular mass of 1.2 MDa by gel-filtration analysis (Thompson et al., 1993). Furthermore, the complex was found to interact with a GST-CTD affinity column (Thompson et al., 1993) as well as with a GST-VP16 affinity column (Hengartner et al., 1995; Thompson et al., 1993). Taken together, this prompted the theory of transcription initiation through recruitment to the promoter of all basic factors and RNAPII as part of a holoenzyme by activators and TFIID (Koleske and Young, 1994).

Another form of holopolymerase was purified from HeLa whole cell extract by affinity chromatography on immobilised recombinant GST-TFIIS (Pan et al., 1997). The isolated complex contained all the general transcription factors, TFIIB, TFIID (TBP1 TAFIIs), TFIIE, TFIIF, and TFIIH as well as RNAPII. Human Cdk8 (yeast Srb10) was also part of the 1.9 MDa complex. The complex was shown to initiate transcription from the adenovirus major late promoter accurately *in vitro*. The biological relevance of holoenzymes containing virtually all the general transcription factors, Mediator, and RNAPII has been questioned by recent results obtained by chromatin immunoprecipitation (reviewed in (Svejstrup, 2004)).

### ***1.9.1 Medc and holoRNAPII***

The isolation by similar chromatographic procedures of two widely different versions of Mediator-RNAPII complex (Kim et al., 1994; Koleske and Young, 1994) raised questions as to whether artefacts due to chromatography, could be influencing the end result. As basis for an alternative purification procedure, Med14 (Rgr1), Med18 (Srb5), Med22 (Srb6) were triple-FLAG tagged and purified by a one step affinity purification procedure from nuclear extract at 250mM KOAc (Liu et al., 2001). Two complexes were isolated: a large complex of an apparent size of 1.9 MDa and a smaller complex of 0.55 MDa. The large complex comprised RNAPII and Mediator, as well as the [Med12, Med13, Cdk8, CycC] module, which was absent from the Kornberg holoRNAPII. This complex was termed polII/Med and purification at higher ionic strength (0.5 M KOAc) disrupted the polII/Med complex, demonstrating salt lability in seeming contrast to purifications of holoenzyme and holoRNAPII where chromatographic steps involved up

to 1.1 M KOAc as well as gel filtration at 0.8 M KOAc (Kim et al., 1994; Thompson et al., 1993). Co-concentration on ion-exchange columns may be a factor in stabilising these large complexes through several purification steps. The smaller 0.55 MDa complex was called Mediator core (or Medc) and consisted of [Med20(Srb2), Med17(Srb4), Med18(Srb5), Med22(Srb6), Med21(Srb7), Med1, Med4, Med6, Med7, Med8, Med11, Med9, Med10(Nut2)]. The abundance of the Medc complex was 20-30% of the polII-Med. in crude nuclear extract (Liu et al., 2001).

### ***1.9.2 Mediator-CTD interaction***

Half of the yeast Mediator Head domain subunits (Med17, Med18, Med20 and Med22) have been identified as Srb proteins (Srb4, -5, -2 -6 respectively) implicating them in RNAPII-Mediator interaction (Nonet and Young, 1989). In support of the genetic data, all the products of the dominant Srb genes (*SRB2*, -4, -5, and -6) were later reported to associate in a sub-complex of Mediator (Lee and Kim, 1998). HoloRNAPII, immobilised by histidine tags on Med14 or Med6 was dissociated by a gradient of urea, revealing the existence of a [Med6, -17, -18, -19, -20 and -22] containing complex and a [Med1, -3, -4, -7,-8, -9, -14(Rgr1), -15(Gal11), -16(Sin4), -21(Srb7)] containing complex. Hence, as the genetic identification of Srb proteins indeed mirrored the physical association of these proteins in Mediator, this pointed to the presence of a CTD-interacting module of Mediator. In a different approach, epitope tagged Mediator subunits were expressed in the baculovirus system and individual Mediator domains were reconstituted by mixing of extracts and purification by affinity chromatography. Head and Middle domains were also immobilised on beads and incubated with a recombinant GST fusion protein carrying five CTD repeats or a negative control consisting of a scrambled consensus repeats. The result from these experiments indicated that residues both in the Mediator middle domain (containing Med9/Med10) and head domain (containing Med17/Srb4) participate in specific CTD binding (Kang et al., 2001). In a different approach, whole cell yeast extract was applied to an anti-CTD immunoaffinity column using the 8WG16 monoclonal antibody. In these experiments, the Mediator tail domain protein, Med15 (Gal11) and head domain proteins Srb2 (Med20), Srb4 (Med17), Srb5 (Med18) and Srb6 (Med22), which are all known to be part of holoRNAPII, were not bound to beads possibly because the association between

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Mediator and RNAPII requires the participation of a free CTD domain (Wade et al., 1996).

The mammalian CTD sequence fused to GST was used as a one-step purification procedure to purify CTD-interacting proteins from HeLa nuclear extract. The analysis revealed a CTD interacting complex that supported activated transcription and was similar to the co-activator CRSP (Taatjes et al., 2002).

### ***1.9.3 Interface subunits in holoRNAPII***

The interaction between Mediator and RNAPII in the holopolymerase isolated in the Kornberg lab is highly dynamic. This was shown by the ability of Mediator (from free holoRNAPII in the supernatant) to dissociate and bind to coreRNAPII immobilised on affinity resin (Svejstrup et al., 1997). Moreover, a purified  $\Delta med2$  version of Mediator, unable to respond to VP16 activator in a defined transcription system, functioned as a dominant suppressor of transcriptional activation of normal holoRNAPII, further indicating Mediator's ability to dynamically interact with holoRNAPII complexes (Myers et al., 1999).

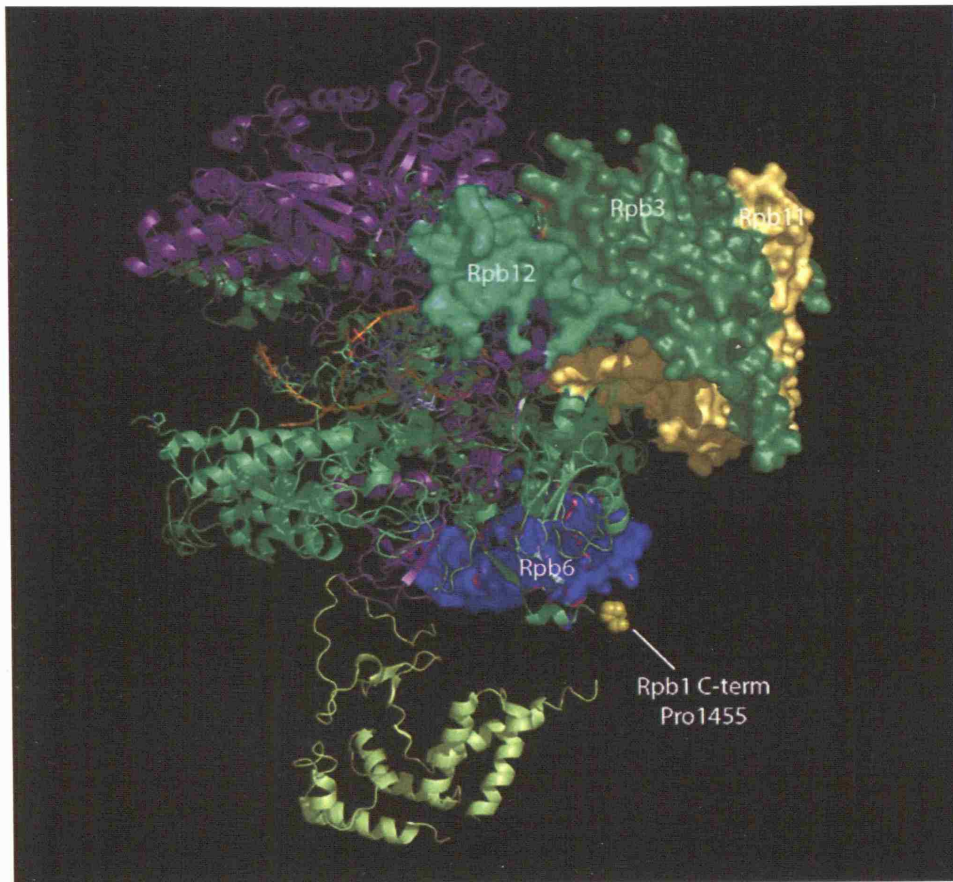


Figure 1 RNAPII structure. Mediator interaction – side view

Shown is Rpb1 (green), -2 (purple), -4 (yellow), -7 in cartoon representation while subunits, reported to interact with Mediator in holoRNAPII, are illustrated in surface representation. Also shown are the DNA strands in orange and the RNA strand in blue. The illustration is based on the structure PDB: 1Y1W. Rpb5, 7, 9 and 10 have been hidden from view for clarity. Created with Pymol (Delano scientific)

Although no crystal structure of holoRNAPII is available, the RNAPII subunits that contact Mediator have been mapped by comparing the *in silico* docked RNAPII/Mediator complex with the structure information obtained from cryoEM analysis of the reconstituted holoenzyme. The interface is created by parts of Rpb1, -2, -3, -6, -11 and -12. The lack of a Mediator crystal structure makes difficult the assignment of specific RNAPII interacting subunits in the Mediator complex. However, the interaction centres on two “flap” regions of the Mediator head domain and two additional regions in the Middle and Middle/Tail domains (Davis et al., 2002).



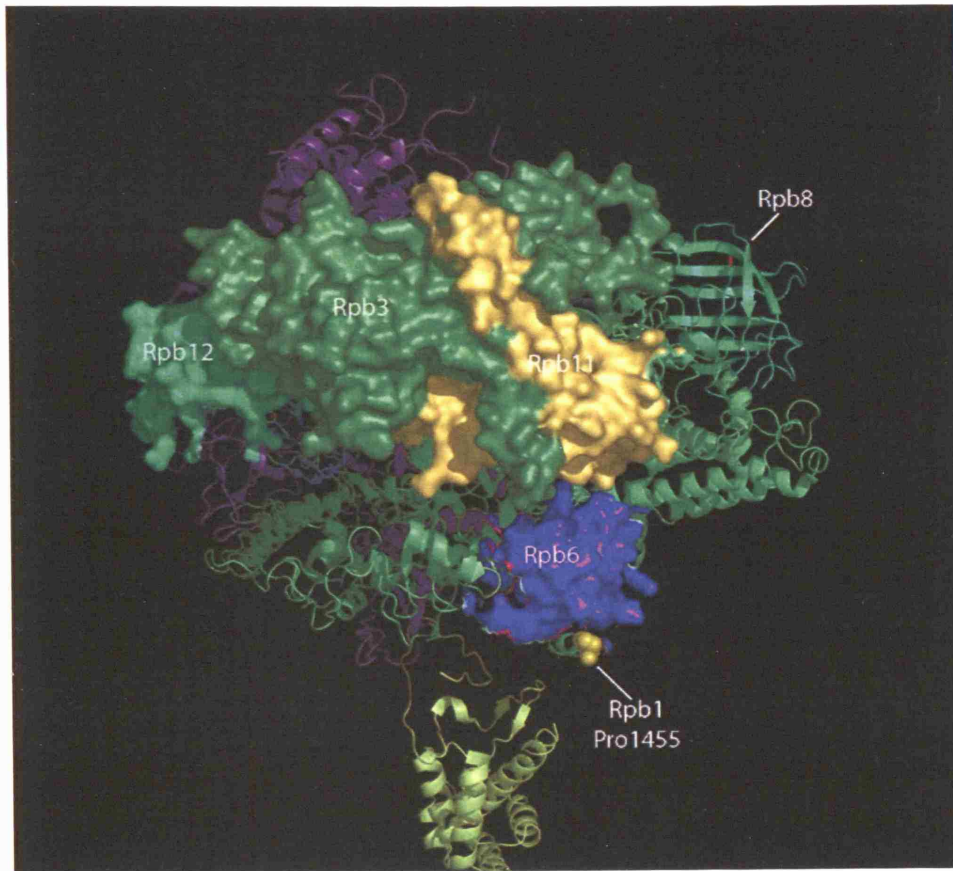


Figure 2 RNAPII structure. Mediator interaction

The coreRNAPII subunits (Rpb3, -6, -11, and 12) reported to be involved in Mediator interaction, are shown in surface representation (see labels). Other subunits (Rpb1, Rpb2) are shown in cartoon representation and Rpb8 is shown in ribbon representation. DNA enters coreRNAPII on the opposite side, making this view a back view of RNAPII. The illustration is based on the structure PDB: 1Y1W. Rpb5, 7, 9 and 10 have been hidden from view for clarity. Created with Pymol (Delano scientific)

#### 1.9.4 Dynamic Mediator-RNAPII interaction

To study the structure of holoRNAPII, Mediator and coreRNAPII were purified to homogeneity and mixed in equimolar amounts. The majority of Mediator complexes (75%) associated with RNAPII to form the holoRNAPII complex and the resulting holoRNAPII complexes were analysed by cryoEM (Asturias et al., 1999).

The results from such analysis indicate that the free Mediator complex is found in a compact shape with the tail domain folded against the middle domain. When comparing the structure of free Mediator with Mediator in the holoRNAPII complex it became apparent that Mediator undergoes a large conformational change as it accommodates intact coreRNAPII. The conformational change depends both on the CTD and the non-CTD coreRNAPII, because when Mediator is challenged with the CTD peptide, no conformational change is observed and only very limited change is seen when Mediator

is mixed with CTD-less core RNAPIIB – and importantly, the Mediator conformation in this case is not as extended as the Mediator in the holoRNAPII complex. The Mediator-RNAPII interaction is also species-specific as yeast Mediator does not bind mammalian RNAPIIA to create a holoenzyme (Davis et al., 2002). These observations imply that RNAPII-Mediator interaction depends on both the CTD and the non-CTD parts of RNAPII.

### 1.10 Mediator recruitment

The recruitment of Mediator to the PIC probably follows different routes depending on the promoter in question. Based on the observation that the majority of Mediator in the soluble fraction is found in complex with RNAPII (Kim et al., 1994), a logical assumption would be that the recruitment of Mediator to a promoter happens concomitant with RNAPII in the form of a holoRNAPII complex. However, a recent time-resolved chromatin immunoprecipitation (ChIP) study of the recruitment of Mediator to the HO locus in yeast, in combination with later data from higher cells, makes this scenario increasingly unlikely. The yeast data showed that the SBF transcription factor was responsible for recruitment of Mediator well ahead of RNAPII (Cosma et al., 2001).

Likewise, the sequence of events after galactose induction of the Gal4-responsive yeast *GAL1* gene promoter was followed. The results indicated that 4-7 min post induction, the SAGA histone acetyltransferase (HAT) complex appears at the promoter. Mediator arrives 2,5 min later and after a further 2.5 min, RNAPII and TBP, TFIIE, TFIIH, and TFIIF arrive simultaneously (within the resolution of the assay: 30sec – 1 min) at the GAL1 promoter (Bryant and Ptashne, 2003). The order of events where TFIIE is recruited after Mediator fits nicely with a role of Mediator in TFIIE recruitment described above (Sakurai et al., 1996).

Furthermore, a minimal PIC comprising RNAPII–IIF–IIB–TBP was recently modelled *in silico* based on cryo-EM structures of holoRNAPII, as well as structural information on the location of TFIIF, TFIIB and DNA in relation to RNAPII. Interestingly, the model predicts that upstream promoter DNA, TFIIB and TBP all are expected to be located at the interface between polymerase and Mediator in the PIC. This implies that RNAPII and Mediator cannot arrive at a promoter as a pre-formed holoRNAPII complex, but must be recruited independently (Asturias, 2004).



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Interestingly, in a *spt20Δ* strain (disrupting SAGA), the timing of Mediator recruitment is unchanged whereas recruitment of RNAPII and the GTF's fails (Bryant and Ptashne, 2003). This may indicate that GAL4 independently contacts SAGA and Mediator (Bryant and Ptashne, 2003), and it suggests that though Mediator is recruited in the absence of Spt20 (or, by extension, SAGA), it is for some reason unable to recruit/accommodate RNAPII at the promoter.

The tail domain of yeast Mediator may have a direct effect on PIC stability as suggested by the observation that PIC's built in nuclear extract (made from either *med16(sin4)Δ* strains or *med3(Pgd1)Δ* strains) are unstable (Reeves and Hahn, 2003).

### **1.10.1 Mediator-activator interaction**

By surface plasmon resonance analysis (Biacore), purified wildtype Mediator analyte was shown to interact strongly with GST-VP16 ligand (and not with a VP16 mutant carrying a non-functional activation domain). Mutant Mediator complexes deficient in Med15 (Gal11) or Med3 (Hrs1/Pgd1) showed negligible interaction with GST-VP16, and the results were similar for interaction with GST-GCN4. These results indicate that Med15 (Gal11) and Med3 (Hrs1/Pgd1) in the Mediator tail domain, constitute a general binding site for acidic transcriptional activation domains (Lee et al., 1999).

Which subunits in Mediator are required for function of VP16 or GCN4 activation was investigated by *in vitro* transcription assays and data indicated that deletion of any one of the tail domain subunits Med2, Med3(Hrs1/Pgd1) or Med16(Sin4) resulted in loss of activation by Gal4-VP16 in a fully defined transcription system while GCN4 activation was only impaired using Mediator devoid of Med16(Sin4) (Myers et al., 1999). The *in vitro* results were supported by similar findings *in vivo* using a Gal4 promoter driving a LacZ reporter and co-expressing the Gal4 DNA binding domain fused to either VP16 or GCN4. These results indicate that VP16 and GCN4 have different targets in the Mediator complex (Myers et al., 1999).

Recently, UV-crosslinking of the Gal4 activator to proteins in PIC's assembled on an immobilised promoter template was investigated in yeast nuclear extract. The results indicated activator-crosslinking to Med15 (Gal11), as well as the TFIID/SAGA component TAF12, and the SAGA subunit Tra1 (Reeves and Hahn, 2005). All in all, observations by several groups suggest that the Mediator tail domain is the main point of contact between yeast Mediator and transcriptional activators.

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Recruitment of Mediator has been suggested to happen through direct contacts between Mediator subunits and the SAGA complex. To investigate further the mechanism of *in vivo* SAGA/Mediator recruitment a fluorescence resonance energy transfer (FRET) based assay was developed where Gal4 was fused to enhanced Cyan fluorescent protein (ECFP), while individual SAGA subunits were fused to enhanced yellow fluorescent protein (EYFP). *GAL1-10* is de-repressed in the presence of galactose and a galactose-dependent FRET signal was indeed observed only when EYFP was fused to the SAGA subunit Tra1 (Bhaumik et al., 2004) indicating that the Gal4 and Tra1 proteins are close together on the promoter. Furthermore, by deletion of individual SAGA subunits, the recruitment of Mediator was shown to depend on a stoichiometric SAGA complex even in Tra1 wildtype strains.

These observations can to some extent be transferred to metazoans. *In vivo* data suggest that the tail domain containing Med15 (ARC105 in human) is responsible for direct interactions with activators such as VP16 in mammalian cells (Mittler et al., 2003). The human TRAP220 subunit (Med1 in yeast) (Bourbon et al., 2004) (which in yeast Mediator is located in the middle domain) is the target for interaction with certain specific transcriptional activators. For example, TRAP220 was reported to be required for transcription activation by the adipogenic activator PPAR $\gamma$ 2. In a mouse embryonic fibroblast cell line devoid of TRAP220, adipogenic differentiation was dependent on over-expression of TRAP220, whereas the myogenic differentiation pathway (which depends on the MyoD activator) was perfectly functional (Ge et al., 2002).

Another example is the Sur2 (Med23) protein, which is a subunit of metazoan Mediator complexes only. When *sur2*<sup>-/-</sup> mouse embryonic fibroblasts were tested in transient transfection assays, GAL4-VP16 activation was at wild type levels. In contrast, two activators (E1A-CR3 and Elk-1) that are at the end of a RTK-Ras-Raf-MEK-ERK signal transduction pathway were unable to activate transcription in these cells. Using immobilised templates in *sur2*<sup>-/-</sup> mouse embryonic fibroblast extracts, a failure to recruit Mediator was shown to be the problem. Co-transfection of a Sur2 expressing construct rescued the activation. These results demonstrate that metazoan Mediator complexes also have specific contact points for activator proteins (Stevens et al., 2002).

### ***1.10.2 CTD or an activator induce Mediator conformation changes***

By cryo-EM analysis of purified yeast and the human Mediator-like complex, TRAP, conservation of overall Mediator structure was demonstrated (Dotson et al., 2000). CryoEM analysis of the interaction between GST-CTD, VP16 and human CRSP revealed a conformational change in CRSP upon incubation with GST-CTD (Naar et al., 2002). Binding of either VP16 or GST-CTD induced similar partial unfolding of CRSP whereas binding of an alternative activator SREBP-1a lead to a different conformation. The binding sites of VP16 and the CTD on Mediator were close, but not overlapping, and centred on the CRSP77 subunit (Med17/Srb4 in yeast), whereas the SREBP-1a activator bound the ARC105 subunit (Med15/Gal11 in yeast). The binding sites mirror activator and CTD binding surfaces in yeast Mediator, where the Med17 subunit is localised in the interface between the Middle and Head domains and the Med15 subunit is part of the tail domain (Guglielmi et al., 2004).

As differences in activator-bound Mediator conformation thus correlated with differences in points of contact with the activator protein, the conformational effects of activators known to contact the same Mediator subunit was investigated. Thyroid hormone receptor (TR) and vitamin D receptor (VDR) were both reported to contact TRAP220 (yeast Med1) (Yuan et al., 1998) which is a component of the human CRSP-Med complex. Data from cryoEM analysis indicated virtually identical changes in Mediator conformation upon TRAP220 binding of TR or VDR. In addition, these changes were distinct from the previous reported changes induced by another activator's contact (Taatjes et al., 2004). Thus, depending on the subunits that comprise a given Mediator ortholog, different activators can induce specific Mediator conformations. Interestingly, the TRAP220 subunit was recently reported to be part of only 20% of human TRAP/Mediator complexes and (in contrast to other human Mediator complexes) RNAPII was in tight association with these TRAP220-containing TRAP/Mediators, creating a human holoRNAPII (Zhang et al., 2005a). Furthermore, this form of TRAP/Mediator was shown to be essential for gene activation by the estrogen receptor (ER) in ER expressing Mcf-7 cells, suggesting a specific rather than general role for the TRAP/Mediator in transcription activation in this context.

Though conformational changes in response to CTD or activator binding have not yet been reported in yeast Mediator, an effect of CTD interaction can not be excluded (Chadick and Asturias, 2005). Furthermore, binding of either VP16 or GST-

CTD to Mediator stimulated activated transcription in a reconstituted transcription system, possibly indicating that Mediator conformational change as such, is sufficient to stimulate initiation or indeed re-initiation (Naar et al., 1998; Naar et al., 2002).

Other mechanisms than activator binding can serve to change the conformation of yeast Mediator. For example, unfolding of mediator could be accomplished by incubation under conditions of pH higher than 8.5 (Asturias et al., 1999). Lowering the pH caused some (but not all) Mediator complexes to return to the compact conformation indicating some irreversible changes might happen in high pH (Asturias et al., 1999). The mechanism of pH mediated conformational change in Mediator is unknown, but it is tempting to speculate that the binding of proteins could bring about a similar change by changing local protein charge.

### ***1.10.3 Mediator and the elongation phase of transcription***

In yeast cells, Mediator exists both on its own and in complex with RNAPII. The predominant form of Mediator is a point of confusion as the abundance of mediator was found to be 6 % of the RNAPII abundance, while 50% of the RNAPII purified was found in complex with Mediator (Kim et al., 1994). The apparent discrepancy was resolved somewhat when it was found that most yeast RNAPII is engaged in active transcription and consequently firmly associated with chromatin *in vivo* (Svejstrup et al., 1997). RNAPII poised at the promoter before initiation can be extracted from chromatin by treatment with 0.5M KOAc. In contrast, the interaction of elongating RNAPII with chromatin template is exceedingly stable, easily enduring extraction with 0.5 M KOAc (Svejstrup et al., 1997). Hence, transcribing RNAPII is absent from the soluble fraction of yeast extracts unless first extracted by extensive sonication of the chromatin and elution in buffer containing 1 M  $(\text{NH}_4)_2\text{SO}_4$  (Svejstrup et al., 2003). Function of the elongation complex (EC) is also very salt tolerant. During work leading up to crystallisation of the elongation complex, coreRNAPII, paused on a immobilised tailed template could be treated with 2M KOAc with a third of elongation complexes still able resume elongation upon nucleotide addition (Gnatt et al., 1997).

The observation that Mediator is only found in promoter regions and not in the coding region of genes, together with a demonstration that RNAPII and Mediator can interact dynamically *in vitro* prompted the hypothesis that Mediator may associate with RNAPII at promoters in a repeated cyclic manner to allow transcription initiation and

re-initiation. Here, CTD phosphorylation might serve to disrupt the interaction, concomitantly allowing RNAPII to escape the promoter and Mediator to be recycled to a new initiating polymerase (Svejstrup et al., 1997).

### 1.11 Basic Mediator functions

Functionally, three different biochemical activities are inherent to the yeast Mediator complex in a reconstituted, fully defined transcription system. First, *in vitro* experiments showed the ability of Mediator to mediate up to 30-fold transcriptional activation (depending on salt concentration) of RNAPII complexes harbouring an intact CTD (Kim et al., 1994). This stimulation is completely dependent on the presence of the CTD, as CTD-less RNAPII does not respond to Mediator or in fact to GAL4VP16 or GCN4 activators *in vitro* (Myers et al., 1998). The strict dependence on the CTD for the activator response would suggest that the activator signal is conveyed through Mediator, which subsequently exerts its effect on RNAPII through the CTD. The second function of Mediator is to stimulate basal (activator-less) transcription 8-fold *in vitro* (Kim et al., 1994). This may imply that Mediator can affect the basic ability of RNAPII to transcribe (Reeves and Hahn, 2003). The stimulation probably depends on coreRNAPII interaction through the head domain of Mediator as analysis of a *srb4Δ* mutant Mediator indicated that it was unable to support basal transcription *in vitro*. Third, Mediator is able to stimulate TFIIH-mediated CTD phosphorylation up to 36-fold (Kim et al., 1994; Myers et al., 1998).

#### 1.11.1 Stimulation of TFIIH kinase activity

Interactions between TFIIH and Mediator are interesting because of Mediator's significant effect on CTD phosphorylation. The TFIIH CTD kinase gene KIN28 is essential for viability, but *kin28ts* strains do exist (Valay et al., 1993). When such a mutation was combined with a *med16(sin4)* mutation, a synthetic lethal phenotype was uncovered (Valay et al., 1995). In addition the TFIIH subunit Tfb1 was shown to interact *in vitro* with GST fused to a region encompassing amino acids 866–929 of the Mediator tail subunit Med15 (Gal11) (Sakurai and Fukasawa, 2000). This data may indicate a direct interaction between holoTFIIH and the Mediator tail domain.

In other experiments, TFIIH and TFIIE were found to interact by Biacore analysis (Bushnell et al., 1996) and interestingly, TFIIE also interacts with the Tail domain subunit Med15 (Gal11) *in vitro*, leading to 5.6 fold stimulation of the TFIIH

CTD kinase activity. Thus, TFIIE may regulate TFIIH kinase activity by bringing together TFIIH and Gal11/Mediator, even though purified mutant Mediator devoid of Gal11 was reported to stimulate CTD phosphorylation to wildtype levels while abolishing *in vitro* activated transcription by Gal4-VP16 (Myers et al., 1999).

Although the experimental proof is still not completely persuasive, the tail domain of Mediator may serve a role in proper association of the PIC components and also, possibly, in stimulation of TFIIH CTD kinase activity.

### **1.11.2 The Mediator head domain and TFIIH kinase stimulation**

The Mediator head domain subunits Med17(Srb4), Med18(Srb5) and Med20(Srb2) are all required for basal and activated transcription *in vitro*, presumably because of a failure in recruitment of RNAPII to the PIC. Deletion of Med20 (Srb2) and Med18 (Srb5) was found to affect the stability, but not the composition, of the purified Mediator complex (Lee et al., 1999; Ranish et al., 1999). This agrees well with the Mediator interaction map (based on yeast two-hybrid and *in vitro* pull down assays) which showed that Med18/Med20 forms a sub-complex of the head domain connected to the rest of mediator via the Med8 subunit (Guglielmi et al., 2004).

To analyse the functions of individual Mediator subunits, the CTD kinase activity of Kin28 towards the CTD in coreRNAPII and in mutant holoRNAPII was compared *in vitro* (Lee et al., 1999). As expected, the TFIIH kinase Kin28 was stimulated 15-fold by the presence of wildtype Mediator, but deletion of either the Med18 or the Med20 subunit abolished Kin28 stimulation, possibly implicating these subunits in CTD interaction (Lee et al., 1999). Interestingly, in the context of the PIC, the GAL4-VP16 and GCN4 activators could also stimulate TFIIH phosphorylation of the holoenzyme (but not coreRNAPII) up to four-fold. Outside of the PIC context, there was no stimulation by the activators suggesting that the PIC creates inter-subunit interactions that are vital for activator-stimulation of Kin28 activity.

### **1.11.3 Mediator-nucleosome interaction**

Purified Mediator was revealed to bind to nucleosomes *in vitro* and to feature histone acetyl transferase (HAT) activity in in-gel HAT assays (Lorch et al., 2000). The non-essential Med5 (Nut1) subunit of Mediator was found to be able to acetylate H3 and to a lesser extent H4 in both nucleosomes in chromatin and histone octamers. The in-gel HAT activity was also a feature of recombinant Nut1 (Lorch et al., 2000). Although the

physiological significance of these results remains unknown, the direct interaction of Mediator with chromatin may point to additional functions in transcription, beside the well-characterized role in co-activation.

### **1.12 CTD and co-transcriptional mRNA processing**

In the above paragraphs, I have delineated some of the events that take place during PIC assembly, transcription initiation and promoter clearance. The CTD probably plays a role both in recruitment of RNAPII as well as in the structural integrity of the PIC. The CTD also plays a major role in orchestrating the events that take place during the transcription elongation and termination phases.

After promoter clearance, RNAPII enters productive elongation phase during which a pivotal role is played by the CTD in the coordination of interaction of RNAPII with factors that help maintain productive transcription (Sims, III et al., 2004). A recent functional screen for CTD binding proteins in yeast extract found specific interaction with proteins known to be involved in transcription, but also a range of other proteins possibly implying additional functions of the CTD domain (Phatnani et al., 2004). The factors interacting with the CTD include mRNA processing factors, such as the capping enzyme machinery (Cheng and Sharp, 2003; Cho et al., 1997), elongation factors such as Spt4 and Spt5 (Lindstrom and Hartzog, 2001), RNAPII ubiquitylation proteins like Rsp5 (Chang et al., 2000), and the human mRNA cleavage and polyadenylation specificity factor (CPSF) (McCracken et al., 1997) in the form of the yeast homologue Yhh1p/Ctf1p (Dichtl et al., 2002b), as well as the related snRNA 3' processing machinery, the Integrator complex (Baillat et al., 2005). These interactions are controlled through reversible covalent modification of the CTD domain, including phosphorylation, dephosphorylation, as well as O-glycosylation. These modifications will be described below.

#### ***1.12.1 Phosphorylation patterns and transcription cycle***

Chromatin immunoprecipitation (ChIP) experiments in *S. cerevisiae* were performed using the phosphorylation specific mAb's H14 to detect Ser5P, and H5 to detect Ser2P. TFIID-dependent Ser5 phosphorylation was detected primarily at promoter regions, while a rapid Ser5 dephosphorylation ensues after promoter clearance. In contrast, significant Ser2 phosphorylation was detected only further into coding regions (Komarnitsky et al., 2000). The specificity of the mAb H5 antibody commonly used to

detect Ser2P has been called into question by Biacore analysis, which indicates that it recognises doubly phosphorylated repeats almost as well as Ser2P-repeats (Jones et al., 2004).

The issues raised regarding specificity of the anti-Ser2P mAb H5, were taken into account in a recent study addressing changes in RNAPII phosphorylation patterns in human pancreatic cells across promoter-, coding-, and intergenic regions of housekeeping genes cyclophilin A (PPIA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the androgen-inducible genes NKX3-1 and PSA (Morris et al., 2005). Using 8WG16, H14, H5 and the N20 antibody raised against the N-terminus of RNAPII, a modified ChIP protocol was employed, and comparison of H14 and H5 signals lead to the conclusion that in human cells, like in yeast cells, Ser5P is abundant in promoter regions, where Ser2P is nearly absent, whereas Ser2P predominates in the 3' regions of the ORF. Additionally, it was suggested that the dephosphorylation of Ser5P during the elongation proceeds slower than previously thought, making it probable that elongating polymerase is doubly phosphorylated to a significant extent. This correlates well with purification from clarified yeast extract using a three-repeat CTD-affinity column purification procedure. The majority of proteins identified specifically bound the doubly phosphorylated CTD while others had preference for either singly phosphorylated Ser5P or Ser2P (Phatnani et al., 2004).

Taken together, the available data suggest that the phosphorylation state of the CTD is probably a major functional determinant in coordination of composition of the transcription elongation complex as it changes dynamically through transcription to signal the current status of affairs.

### 1.13 CTD phosphorylation

A number of kinases in the yeast proteome can phosphorylate the CTD repeats, but only a few kinases show genetic interaction with elongation factors. The kinase complexes known to be involved in transcription in yeast are Kin28-Ccl1 (associated with TFIIF in TFIIFK), Srb10-Srb11, Ctk1-Ctk2 and Bur1-Bur2. These CTD kinases all belong to the family of Cdc2-like cyclin-dependent kinases. The repeat sequence {Tyr1, Ser2, Pro3, Thr4, Ser5, Pro6, Ser7} of the CTD contains three serine residues of which Ser2 and Ser5 are targets *in vitro* by the cyclin dependent kinases Kin28, CTDK-1, Srb10 and Bur1, whereas S7 is targeted by the kinase DNA-PK (Trigon et al., 1998). The functional role of CTD Ser7 phosphorylation, if any, is currently unknown.



## Introduction: CTD phosphorylation

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The Y1 position is phosphorylated by c-abl in HeLa cells, and this has been correlated with DNA damage and cell cycle progression rather than transcription. The extreme C-terminus of the CTD in mammals additionally encodes a recognition site for the C-abl kinase as well as Casein kinase II (Baskaran et al., 1993; Chapman et al., 2004; Oelgeschlager, 2002). The importance of these sites is presently unclear.

### **1.13.1 TFI<sub>IK</sub>**

During purification of yeast initiation “factor b” (or TFI<sub>IIH</sub>), a processive CTD kinase activity inherent to the complex, was uncovered by SDS-PAGE analysis of time course studies of a phosphorylation induced band-shift of RNAPII. Even at very early time points no intermediate bands between the shifted RNAPII<sub>0</sub> and non-shifted RNAPII<sub>A</sub> was observed, indicating a processive mode of action (Feaver et al., 1991). Through competition experiments using an excess of unlabelled competitor nucleotides, the TFI<sub>IIH</sub> kinase was shown to have a substrate preference for ATP or dATP whereas it could not utilize GTP, CTP or UTP (Feaver et al., 1991). The kinase activity was later found to reside in the TFI<sub>IK</sub> sub-complex, consisting of the kinase Kin28, its cyclin, Ccl1 and an additional subunit Tfb3. All subunits of the TFI<sub>IK</sub> complex are essential for viability in yeast and using a *ts* allele of *kin28* in a genome-wide microarray analysis (at permissive and restrictive temperature) showed that the CTD kinase subunit is essential for transcription of most yeast mRNA genes (Holstege et al., 1998), and see [http://jura.wi.mit.edu/young\\_public/factors/KIN28/KIN28\\_grid.html](http://jura.wi.mit.edu/young_public/factors/KIN28/KIN28_grid.html)).

A metazoan complex homologous to (Kin28/Ccl1/Tfb3) comprises Cdk7/CyclinH/Mat1, (Feaver et al., 1997), which additionally serves a role in the cell cycle, where it acts as a cyclin-dependent kinase activating kinase (CDK-activating kinase or CAK) (Serizawa et al., 1995). In contrast to its human orthologue, TFI<sub>IK</sub> failed to activate human CDK2-cyclin *in vitro* (Feaver et al., 1994). A different kinase, Cak1, was shown to be responsible for the activation of the major cell cycle kinase, Cdc28 in yeast (Kaldis et al., 1996; Thuret et al., 1996).

### **1.13.2 Substrate specificity**

Kin28/Ccl1 is a Ser5-specific CTD kinase, and by comparing a wild type strain to a *kin28-ts* strain it was shown to be responsible for the majority of phosphorylation of Rpb1 in total yeast extract (Valay et al., 1995). TFI<sub>IK</sub> does not depend on coreTFI<sub>IIH</sub> for activity and is able to phosphorylate the RNAPII CTD using dATP even when the

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ATP-dependent helicase activity of TFIIH is inhibited by addition of ATP $\gamma$ S (Jiang and Gralla, 1995). Interestingly, by far western Kin28 was shown to interact specifically with Rpb1 and Rpb2 (Feaver et al., 1994) whereas measured by Biacore analysis no stable interaction between purified RNAPII and TFIIH could be detected (Bushnell et al., 1996).

Additional phosphorylation targets in the PIC are the Med4 and Med14 (Rgr1) Mediator subunits (Liu et al., 2004). Mutation of the phosphorylation site in Med4 caused no detectable phenotype, suggesting that no essential role is played by this phosphorylation event (Guidi et al., 2004).

### 1.13.3 CTDK-1

The C-terminal domain kinase 1 (CTDK-1) is the putative yeast homolog of the mammalian Cdk9, also called positive elongation factor b (P-TEFb). Genetic interactions with *SPT4*, -5 and Elongator indicate a role in elongation (Lindstrom and Hartzog, 2001), and chromatin immunoprecipitation experiments (ChIP) in a *ctk1Δ* mutant strains show effects on the recruitment of the polyadenylation machinery to the ORF of active genes (Ahn et al., 2004). CTD kinase 1 (CTDK-1) is a complex of three members: Ctk1, the 60.5 kDa cyclin dependent kinase subunit, Ctk2, its 37.9 kDa cyclin, and Ctk3, a 34.8 kDa protein, which is essential for Ctk2 activation of Ctk1 (Hautbergue and Goguel, 2001). CTDK-1 is non-essential, but cells deleted for *CTK1* are slow growing and are inviable at 15°C (Lee and Greenleaf, 1991). CTDK-1 is the main CTD Ser2 kinase *in vivo* (Cho et al., 2001), but is reported to be able to phosphorylate both Ser2 and to some extent, Ser5 *in vitro* (Jones et al., 2004). Its action is probably processive, giving rise to only one hyperphosphorylated RNAPII band upon 5% SDS-PAGE, and phosphorylation follows two kinetic phases with a lag phase followed by a “burst”-phase (Morris et al., 1997). This behaviour might be explained by the observation that repeats that are pre-phosphorylated on Ser2 or Ser5 become even better substrates for Ctk1 (Jones et al., 2004). Biochemical characterisation of CTDK-1 activity towards a purified GST-CTD fusion protein found a requirement for Mg<sup>2+</sup> (> 1 mM) and  $K_m = 27 \mu\text{M}$  ATP. Furthermore, CTDK-1 activity was unaffected by Ca<sup>2+</sup> concentration or presence of calmodulin (Lee and Greenleaf, 1989), observations which are relevant to this work as both conditions are encountered during the TAP tag

purification procedure which was used to purify the CTDK-1 complex in this work (see results section).

CTDK-1 associates physically with the elongating polymerase, balancing the action of associated CTD phosphatases (Cho et al., 2001). Remarkably, Cak1 activates both TFIIF and CTDK-1 by phosphorylation of Thr-162 in the T-loop in the CTD kinase Kin28 (in TFIIF) (Espinoza et al., 1998), and by phosphorylation of Thr-338 in the T-loop in Ctk1 (in CTDK-1) (Ostapenko and Solomon, 2005). The basis of the activation was suggested to be to increase the affinity of the kinase for its cyclin (Keogh et al., 2002).

### **1.13.4 Cdk8 (Srb10)/CycC (Srb11) kinase**

The “Suppressor of RNAPB” (Srb) genes were initially identified based on their ability to suppress the effects of CTD-truncation mutations (Nonet and Young, 1989), and, furthermore, the Sca1 (Suppressor of CTD alanine) gene, which is isoallelic with the gene coding for Med13 (Srb9), was isolated in a genetic screen for suppressors of CTD substitution mutations that changed the consensus Ser2 or Ser5 positions to alanine. Remarkably, a *med13Δ* mutant was able to suppress the effects of mutation of Ser2, but not Ser5 of the CTD repeat, separating the effect of the two phosphorylation patterns in relation to Med13(Srb9) function (Yuryev and Corden, 1996).

By genome wide expression analysis, the Srb10 kinase was found to be functionally associated with transcriptional repression of 173 genes (see [http://jura.wi.mit.edu/young\\_public/factors/SRB10/SRB10\\_grid.html](http://jura.wi.mit.edu/young_public/factors/SRB10/SRB10_grid.html) and (Holstege et al., 1998)). The mechanism of transcriptional repression by the [Med12, -13, Cdk8, CycC] complex has been suggested to be to phosphorylate the CTD of incoming coreRNAPII hindering its untimely incorporation in the PIC in yeast (Hengartner et al., 1998) and in human (Sun et al., 1998). In addition, it was shown that human Cdk8 negatively affects transcription by phosphorylation of Cdk7 (homologue of Kin28) in the TFIIF complex (Akoulitchiev et al., 2000). Other observations show that the inherently unstable activator protein Gcn4, is a target for the Cdk8 (Srb10) kinase. Furthermore, the phosphorylation serves to mark Gcn4 for ubiquitylation and subsequent degradation by the proteasome pathway. In a *cdk8(srb10)Δ* strain, the stability of Gcn4 was significantly improved relative to a wt Cdk8 strain, indicating that the Cdk8-mediated degradation pathway is a major regulator of Gcn4 in the yeast cell, and that the degradation of Gcn4 might be

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the way the [Med12, -13, Cdk8, CycC] complex exerts its repressive function (Chi et al., 2001).

### 1.13.5 Substrate specificity of the Cdk8 kinase.

Purified [Med12, -13, Cdk8, CycC] complex was used to phosphorylate a GST-CTD fusion protein. The complex is able to phosphorylate both Ser2 and Ser5 of the CTD repeats *in vitro* (Borggreffe et al., 2002). The kinetics of Cdk8 CTD phosphorylation probably are distributive, as intermediate band-shifts were apparent in SDS-PAGE of the phosphorylated GST-CTD (Borggreffe et al., 2002). Genetic engineering of the Cdk8 substrate binding pocket allowed the kinase to utilize a labelled ATP analog ([ $\gamma$ -<sup>32</sup>P]N<sup>6</sup>-benzyl-ATP), which made it possible to identify two additional targets (yeast TFIID components Bdf1 and TAF2) in a PIC assembled in nuclear extract (Liu et al., 2004). Cdk8 also interacts with and phosphorylates Ser208 of the Med2 subunit of Mediator. Mutation of the phosphorylation site to alanine (*med2-S208A*) specifically de-represses the expression of a subset of genes that are normally induced by the transcription factor Rcs1/Aft1 under conditions of low iron (Hallberg et al., 2004; van de Peppel et al., 2005). This directly illustrates the function of Mediator as a modulator of the signal from Rcs1/Aft1 to the basal transcription machinery.

### 1.13.6 *Bur1-Bur2*

*BUR1* and *BUR2* (bypass UAS Requirement) genes were identified in a screen in yeast for mutations that could suppress the requirement for an upstream activator sequence, deleted from the *SUC2* gene promoter. The gene was found to encode an essential Cdc28 related kinase (Bur1) and its cyclin Bur2 (Prelich and Winston, 1993). Affinity tagged Bur1/Bur2 can pull down RNAPIIA from crude extracts, indicating that Bur1/Bur2 prefers to bind to the hypo-phosphorylated CTD and by immunodetection with the H14 antibody, the target specificity was reported to be Ser5 of the heptapeptide repeat. Bur1 mutations give sensitivity to the nucleotide analog (and transcriptional inhibitor) 6-azauracil (6AU) and strong synthetic growth phenotypes were observed with in combination with mutations in the elongation factors Spt4/Spt5, Ctk1 and a CTD-truncation mutant (Lindstrom and Hartzog, 2001; Murray et al., 2001), together suggesting a role for Bur1 in transcription. By ChIP, Bur1/2 were shown to cross-link to actively transcribed genes and also to genomic regions 3' to the polyadenylation signal (Keogh et al., 2003). Similarly to Kin28 and Ctk1, Bur1 is phosphorylated by the

Cak1 kinase, and the Bur complex was recently reported to be important for Paf1 recruitment to the transcription complex (Laribee et al., 2005), creating a possible basis for the transcriptional defects in *bur1/2Δ* strains.

## 1.14 CTD dephosphorylation

### 1.14.1 TFIIF associated CTD phosphatase 1 (FCP1)

Dynamic CTD phosphorylation is achieved through the constant interplay between kinases and CTD-specific phosphatases. The first CTD-specific phosphatase to be discovered was the TFIIF-associated CTD Phosphatase, Fcp1 (Chambers and Dahmus, 1994) belonging to class C of phosphatases sharing a DXDX(T/V) motif at the active site. Fcp1 assembles into the RNAPII elongation complex and its activity is stimulated by TFIIF. In *S. pombe*, the Fcp1 phosphatase has been reported to interact directly with the RNAPII subunit, Rpb4, and is part of a RNAPII/TFIIF/Fcp1 complex, which can be isolated using Rpb3- or Fcp1-affinity chromatography (Kimura et al., 2002). Repression of *S. pombe* Rpb4 expression correlates with an increase in the proportion of the IIO form in Rpb3 affinity-tag purified RNAPII, further suggesting that Rpb4 is necessary for RNAPII-Fcp1 interaction which helps keep RNAPII in the hypophosphorylated state (Kimura et al., 2002). The Rpb4/Rpb7 complex from *S. cerevisiae* was also shown to associate with Fcp1 (Kamenski et al., 2004). Fcp1 travels with the elongating polymerase as it traverses the coding sequence (Cho et al., 2001; Kong et al., 2005). *In vivo*, Fcp1 is associated specifically with dephosphorylation of Ser2 in the CTD during elongation (Cho et al., 2001), though *in vitro*, it prefers to act on Ser5P (Kong et al., 2005) making its substrate preference a point of some uncertainty. Using a defined transcription system purified from HeLa cells, recombinant Fcp1 (expressed in the baculovirus system) was demonstrated to stimulate transcription in a way distinct from purified TFIIF (Mandal et al., 2002). TFIIF optimally stimulated transcription in catalytic amounts (relative to RNAPII) while stoichiometric amounts of Fcp1 were required for optimal transcription stimulation. This could indicate that Fcp1 binds to RNAPII and alters it sterically in some way that stimulates activity. Support for this model comes from early work suggesting that Fcp1 binds RNAPII at a site separate from the CTD (Chambers et al., 1995) and recent more recent work showing that the catalytic activity of Fcp1 towards the CTD is unaffected by this binding (Suh et al., 2005). Furthermore, human Fcp1 has been shown to stimulate transcription elongation

*in vitro* independently of the phosphatase activity (Mandal et al., 2002). On the other hand, Fcp1 has also been reported to be inhibited by incorporation of RNAPII into a ternary complex *in vitro* (Kong et al., 2005) suggesting either that changes in RNAPII conformation can affect Fcp1 activity or alternatively, that Fcp1 cannot properly access the CTD in the ternary complex.

### **1.14.2 Small cellular Phosphatases**

Based on sequence homology to FCP1, the gene encoding the human Small Cellular Phosphatase 1 (Scp1) was isolated and cloned (Yeo et al., 2003). Scp1 is a member of a family (Scp1–3) of phosphatases that are specific for Ser5P repeats *in vitro*. The X-ray crystallographic structure of Scp1 and its interaction with a CTD repeat peptide showed that the structure of the active sites of Fcp1 and Scp1 are similar (Kamenski et al., 2004). The result of knock down of expression of *Drosophila* SCP (dSCP), using siRNA interference was a specific de-repression of expression of neuronal genes (Yeo et al., 2005), thus indicating a gene-specific role and, presently, the general importance of these putative CTD phosphatases (if any) is unclear.

### **1.14.3 Ssu72**

The essential Ssu72 protein was reported to affect start site selection and to interact with TFIIB (Sun and Hampsey, 1996). It was also found to be a component of the yeast cleavage/polyadenylation factor (CPF), and to interact genetically and physically with the Pta1 subunit of CPF (Dichtl et al., 2002a; He et al., 2003).

Recombinant yeast Ssu72 is a Ser5 specific CTD phosphatase *in vitro*, and degran-mediated depletion of Ssu72 results in an increase in global Ser5 phosphorylation *in vivo*, indicating that Ssu72 is the major Ser5 phosphatase in yeast (Krishnamurthy et al., 2004).

Using purified Ssu72 and differently phosphorylated CTD-peptide substrates, the residues important for Ssu72 recognition were delineated through a detailed kinetic analysis. Results indicated that Ssu72 specifically recognises Thr4 and Pro6 as well as Tyr 1 in the adjacent heptad repeat, fitting with the notion of the general importance of di-heptads mentioned above (Stiller and Cook, 2004).

Temperature sensitive *Ssu72-ts* mutants failed to produce correctly 3' cleaved mRNA in yeast extracts at the restrictive temperature, but polyadenylation was not significantly

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affected, indicating that, although part of CPF, Ssu72 probably plays a role only in 3' mRNA cleavage (He et al., 2003).

A GST-Ssu72 fusion protein was able to specifically pull down a HA tagged Kin28 protein from yeast crude extract (Ganem et al., 2003), and, indeed, Ssu72 was also reported to interact with RNAPII through co-immunoprecipitation of RNAPII with S<sup>35</sup>-labelled, *in vitro* translated Ssu72 (Pappas, Jr. and Hampsey, 2000). Furthermore, by the reverse experiment, Ssu72 was reported to interact *in vitro* with Rpb2 (Dichtl et al., 2002a). The growth defect of temperature sensitive mutants of Ssu72 could be rescued by addition of the uracil analogue 6-azauracil (6-AU), which depletes cellular GTP pools and slows down transcription (Dichtl et al., 2002a). Taken together, these observations suggest a tight link of Ssu72 activity with transcription and CTD function.

### 1.15 Other CTD modifications

Other modifications besides phosphorylation have been reported to change the CTD. Indeed, inspired by the so-called “histone code” describing the multiple modifications of N-terminal histone tails, the phrase “The CTD code” was coined to describe likewise covalent modification of the CTD (Buratowski, 2003).

#### 1.15.1 CTD O-Glycosylation

When RNAPII was purified from calf thymus, O-linked N-acetylglucosamine (O-GlcNAc) was found to be associated with RNAPIIA (but not RNAPIIB or RNAPIIO), indicating that the CTD is the target for this modification, and also that only RNAPIIA becomes glycosylated (Kelly et al., 1993). O-glycosylation, like phosphorylation, is a dynamically reversible modification catalysed by the enzyme O-glycosylase transferase (OGT) and in fact, the two modifications are mutually exclusive events (Kamemura et al., 2002). The biological significance of this modification also remains to be determined.

#### 1.15.2 CTD Isomerisation

Ess1 is the essential yeast homologue of the human Pin1 proline isomerase belonging to the parvulin family of peptidyl-prolyl cis/trans isomerases (PPI). Genetic interactions in *S. cerevisiae* between Ess1 and the transcription related CTD kinases Kin28, Ctk1, Burl and Srb10 have been reported. Ess1 has been shown to interact with the RNAPII0 CTD *in vitro* (Morris et al., 1999; Wu et al., 2000). By circular dichroism analysis of CTD-

peptide binding to the Ess1 WW domain, it was shown that while Ess1 has negligible affinity for an unphosphorylated CTD peptide, it preferentially binds a doubly phosphorylated CTD repeat peptide (Myers et al., 2001). The preference for doubly phosphorylated CTD repeats may point to a role of Ess1 after promoter clearance as the CTD on the promoter is predominantly singly phosphorylated. Using NMR analysis on peptides containing Ser-Pro bonds, the rate of spontaneous *cis*-to-*trans* isomerisation was almost 9 times higher than the *trans*-to-*cis* rate constant (Reimer et al., 1998), which points to a possible role for catalysis of the isomerisation reaction in regulation of CTD-binding of those proteins that discriminate between *cis* and *trans* proline bonds. The mRNA 3' end processing factor, Pcf11 for example, was reported to have a CTD isomer preference. In the crystal structure of a CTD peptide bound to the Pcf11 CTD-interaction domain (CID) the S-P bonds were in the *trans* configuration illustrating isomer preference (Noble et al., 2005). The precise physiological role of Ess1 in CTD biology and transcription remains unclear.

### 1.16 Post-initiation events

Recruitment of the machinery that modifies the 5' end of nascent eukaryotic pre-mRNA to contain a special m<sup>7</sup>GpppN cap structure is coordinated by the change in the phosphorylation state of the CTD during promoter clearance (Schroeder et al., 2000). In *S. cerevisiae*, the capping machinery consists of the mRNA capping enzyme, the Ceg1/Cet1 complex (Itoh et al., 1984b), and the cap methyltransferase, Abd1. Using ChIP, a number of *abd1-ts* mutant strains were characterised at the permissive and non-permissive temperatures for the occupation of RNAPII on a collection of model ORF's (*ACT1*, *TEF1*, *ENO2* and *PGK1*) (Schroeder et al., 2004). Abd1 was found to have a role both in the stabilisation of RNAPII interaction with the PIC and also in supporting successful promoter clearance and dephosphorylation of CTD at Ser5P, pointing to an important role for the protein, both pre- and post-initiation, possibly constituting a check-point for successful mRNA capping before the RNAPII is released into the coding sequence. A similar conclusion was reached after experiments in a fully defined human transcription system, where RNAPII was halted at different positions and capping enzyme (CE) was added with radioactive GTP to enable detection of capping (Mandal et al., 2004). The results suggested that 20 - 24 nucleotide nascent RNA was rapidly capped whereas RNA of only 16 nucleotides was not, consistent with the length of the mRNA exit tunnel (18 nucleotides) measured by nuclease protection assays (Gu



et al., 1996) and further indicating that mRNA capping occurs as soon as the nascent mRNA reaches the surface of RNAPII.

Cet1 is a RNA 5'-triphosphatase that catalyses the removal of the 5'  $\gamma$ -phosphate of the nascent pre-mRNA, while Ceg1 is a guanylyltransferase that catalyzes the transfer of the GMP moiety from GTP to the 5' end of the RNA, creating the cap structure (5'GpppN-) (Itoh et al., 1984a). Capping is finalized by Abd1, which transfers a methyl group from S-adenosylmethionine to the cap structure, resulting in mRNA containing an N7-methylguanine cap (Schwer et al., 2000). Both Abd1 and Ceg1 specifically interact with the Ser5 phosphorylated CTD and this interaction depends on Kin28 kinase *in vivo* (Rodriguez et al., 2000; Schroeder et al., 2000). Furthermore, Abd1 has been shown to interact with Rpb2 and the elongation factor Spt5 by affinity chromatography (Gavin et al., 2002). ChIP assays indicate that whereas Abd1 travels together with the elongation complex (EC) to the end of a gene, Ceg1 gradually dissociates from the EC as the level of CTD Ser5 phosphorylation decreases. Interestingly, dissociation of Abd1 at the 3' end of an ORF also depends on the action of the phosphatase Fcp1 (Schroeder et al., 2000).

Together, the human DRB sensitivity inducing factor (DSIF), consisting of the human homolog of yeast elongation factors Spt4/Spt5 (Wada et al., 1998a) and the negative elongation factor (NELF) (Yamaguchi et al., 1999), inhibited promoter escape *in vitro*, but this inhibition was relieved by CE addition, suggesting that CE/DSIF/NELF together function to create a promoter escape checkpoint for successful capping of nascent mRNA (Mandal et al., 2004). The observations that a Ceg1 mutant is sensitive to the nucleotide analog 6-azauracil and that synthetic lethality occurs in combinations of *ceg1Δ* and *rpo21* elongation defect mutants, may imply an *in vivo* role for Ceg1 in transcription elongation (Kim et al., 2004a).

### ***1.16.1 Transcription Elongation***

Elongation complexes, assembled in a crude yeast nuclear extract, were halted by addition of terminating 3-O-methyl-GTP nucleotides, after synthesis of 380 base pairs of a immobilised template. By gel-filtration analysis of washed and eluted complexes, the size of the EC was estimated to be between 1 MDa and 1.2 MDa (Pardee et al., 2003), suggesting, at least, that the complex consists of much more than the 520 kDa molecular weight of yeast coreRNAPII (Bushnell et al., 2004). Indeed, ChIP analysis

results indicate that throughout its journey in the coding sequence, the transcription elongation complex is a dynamically changing entity, as CTD-kinases and -phosphatases (Cho et al., 2001), the mRNA capping apparatus, as well as cleavage and polyadenylation factors (Ahn et al., 2004; Bentley, 2005) associate with, and disassociate from, elongating coreRNAPII.

Yeast transcription elongation factors include Spt4/Spt5 (human DSIF) (Hartzog et al., 1998) and Spt16/Pob3 (human FACT) (Lindstrom et al., 2003), the yeast TFIIS factor (DST1) (Wind and Reines, 2000) as well as the Polymerase Associated Factor Paf1 complex (Shi et al., 1996; Squazzo et al., 2002). Genetic evidence showing strong synthetic phenotypes in strains with truncated RNAPII CTD *spt4Δ*, *spt5Δ* and *ppr2Δ* (TFIIS) indicates that the CTD plays a crucial role in coordinating the dynamic association of these many factors with RNAPII (Lindstrom and Hartzog, 2001).

### ***1.16.2 Chromatin remodelling and transcript elongation***

The need for chromatin remodelling activity is not confined to promoter regions as chromatin structures also impede the progress of the transcription complex *in vitro* (Izban and Luse, 1991). *In vivo*, factors exist that help to remove obstacles to transcription elongation (Svejstrup, 2002). Chromatin immunoprecipitation (ChIP) analysis of the nucleosome occupancy in coding sequences of actively transcribed genes have shown that passing of a transcription elongation complex leads to nucleosome loss from the template on some genes. These observations suggest that transcription and chromatin remodelling might be interconnected processes in general (Kristjuhan and Svejstrup, 2004). Remodelling that causes loss of nucleosome octamers from DNA is distinct from sliding nucleosomes along the DNA, which can presumably only be utilised effectively in regions of lower density of nucleosome occupation (Studitsky et al., 2004; Svejstrup, 2004). A human complex that facilitated chromatin transcription (FACT) *in vitro* was isolated (Orphanides et al., 1998). Human FACT was shown to remove one H2A-H2B dimer from the histone octamers upon transcription *in vitro* (Belotserkovskaya et al., 2003) and additionally, to harbour histone chaperone activity which might reset the nucleosome after passing of the transcription complex. The yeast homolog of FACT is Spt16/Pob3, which was reported to co-purify with the elongation factor Spt5 and by implication, with transcribing RNAPII (see below) (Lindstrom et al., 2003).

## Introduction: Post-initiation Events

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Some elongation factors travel with RNAPII through the coding sequence while others may only associate with the EC once it becomes stalled. Thus, by ChIP analysis, gene activation results in the recruitment of Spt5 and Paf1/Cdc73 to the coding sequence of the GAL1 and TEF1 genes, while similar recruitment of TFIIS was correlated with 6-AU treatment, which depletes cellular levels of GTP and UTP causing RNAPII to pause (Pokholok et al., 2002). Pausing increases the risk of spontaneous backtracking of RNAPII which will displace the 3' end of the nascent mRNA strand from the active site. The 3' of the RNA then enters the nucleotide entry funnel in the RNAPII structure (Kettenberger et al., 2004). TFIIS has been shown *in vitro* to stimulate the nuclease activity of RNAPII to cleave nascent mRNA, several bases from the 3' end of the transcript (Izban et al., 1995). This serves to realign the 3'-end of mRNA with the active site of RNAPII to enable continued transcription. In addition, TFIIS may influence the conformation of the elongation complex to make it more processive (Kettenberger et al., 2004).

Spt4 and Spt5 were originally isolated in a yeast genetic screen for suppression of the effects of insertion of a Ty transposon in the promoter of the yeast HIS4 trophic marker gene (Winston et al., 1984). A complex of Spt4/Spt5 was later isolated from yeast (Hartzog et al., 1998) and shown to stimulate the rate of transcription elongation *in vitro* (Wada et al., 1998b). As described above Spt4/spt5 may also participate in a mRNA capping checkpoint at promoter clearance (Mandal et al., 2004).

Purification of the elongating RNAPII and associated factors from yeast chromatin, found the six-member Elongator complex associated with elongating RNAPII (Otero et al., 1999). In bandshift assays, the purified Elongator complex binds specifically both DNA and RNA, with a preference for RNA (Gilbert et al., 2004; Winkler et al., 2002), and by RNA-IP, Elongator interaction with un-spliced nuclear pre-mRNA *in vivo* can be detected (Gilbert et al., 2004). The Elp3 subunit harbours HAT activity and although its non-essential, double-mutant yeast strains with point mutations that disable the HAT activity in Elp3 and the major HAT Gcn5, reveal a very severe synthetic growth phenotype (Wittschieben et al., 2000). A complex, orthologous to yeast Elongator, was isolated from human cells and shown to facilitate elongation through a chromatin template *in vitro*, in an acetyl-CoA dependent manner indicating a role for the HAT activity in the stimulation of elongation (Kim et al., 2002).

### ***1.16.3 Cleavage and polyadenylation factors***

RNAPII and its CTD are closely connected to the process of pre-mRNA maturation. Disruption of the CTK1 gene in yeast results in defects in recruitment of polyadenylation factors (Ahn et al., 2004)

By large-scale affinity purification (using a TBP monoclonal antibody) from HeLa nuclear extracts, the holo-TFIID complex was purified and shown to include the three subunits of the cleavage and polyadenylation specificity factor (CPSF) previously identified (Bienroth et al., 1991). A defined transcription system was used to assemble PIC's. By immunoprecipitation of RNAPII before transcription, CPSF as well as the TFIID complex, was observed to co-precipitate. After transcription initiation, only CPSF could be co-precipitated with RNAPII indicating that at this point it leaves the PIC to join RNAPII during transcription. Further experiments showed that CPSF associates with pre-mRNA (Dantonel et al., 1997).

Prompted by the finding that in HeLa cells, RNAPII CTD truncation mutants cause reduction in properly poly-A terminated transcripts, HeLa cell extract was fractionated over GST-CTD resin with or without hyperphosphorylation of the CTD. The CPSF factor could be purified on a GST-CTD affinity column and was reported to bind to the RNAPII CTD in both hypo- and hyperphosphorylated states (McCracken et al., 1998). The model proposed was of CPSF binding to RNAPII in a position where it is able to read the emerging sequence and react when the polyadenylation signal AAUAAA emerges from RNAPII. The essential yeast gene Brr5/Ysh1 was isolated based on its sequence homology to human CPSF-73 (Chanfreau et al., 1996).

Other mRNA processing complexes associate with the CTD. Recently, a ~12 subunit complex termed Integrator (INTEgrating the CTD of RNAPII largest subunit with the 3' end processing of small nuclear RNAs U1 and U2) was isolated from a HeLa cell nuclear extract (Baillat et al., 2005). The Int1 and Int9 subunits were found to be highly homologous to the human CPSF-73 subunit. The purified Integrator was found to associate *in vitro* with the CTD (in RNAPII and in GST-CTD) and by ChIP to be associated with the genes encoding U1 and U2 small nuclear RNA. By RNA interference knock-down of Int1 or Int11 a accumulation of the primary transcripts of the U1 and U2 snRNA was observed, suggesting a role for Integrator in 3' end processing of snRNA U1 and U2.

#### ***1.16.4 Specific ubiquitylation of RNAPII***

In the elongation phase, specific ubiquitylation factors can mark stalled RNAPII for removal and degradation through the proteasome pathway. This may serve to free up essential reading frames that would otherwise be blocked by an irreversibly stalled RNAPII (Svejstrup, 2003). Rsp5 has been found to direct specific ubiquitylation of RNAPII, being the last step in the E1-E3 ubiquitylation pathway (reviewed in (Pickart, 2001)). Originally, the essential *RSP5* allele was isolated in a genetic screen for mutations that Reverses the Spt Phenotype (Rsp5) (Grant et al., 1997). Rsp5 receives activated ubiquitin from an E2 enzyme and transfers it to RNAPII (Beaudenon et al., 1999; Huibregtse et al., 1997).

Rsp5 was shown to interact with the RNAPII CTD (with a preference for the IIA form) (Chang et al., 2000; Morris and Greenleaf, 2000). *In vitro* ubiquitylation experiments on RNAPII loaded onto DNA templates uncovered little inhibition by Ser2 phosphorylation, but complete inhibition by Ser5 phosphorylation (Somesh et al., 2005). Interaction of Rsp5 with RNAPII may not be exclusively mediated by the CTD, as a recent high throughput screen for Rsp5-interacting proteins identified the RNAPII subunit Rpb7 (Kus et al., 2005). Interestingly, the RNAPII crystal structure shows Rpb7 to be close to where the CTD is linked to the main RNAPII body (Armache et al., 2005), which may indicate that the Rsp5 interaction surface *in vivo* is a combination of these two regions of RNAPII.

#### ***1.16.5 Transcription termination***

##### **1.15.6 Interaction with Pcf11 and RNA causes template dissociation**

The biochemical mechanism underlying transcriptional termination remains one of the ‘final frontiers’ for the research into the transcription cycle. The EC actually transcribes through the polyadenylation signal and into the 3’ UTR. A transition in the composition of the EC at this point has been detected by ChIP. A strong decrease in the level of Ser2P and the departure from the EC of the Ctk1 and Bur1 kinases has been detected by ChIP (Kim et al., 2004b).

Recently, it was shown that the release of RNAPII from the DNA template depends on the presence of both RNA and the CTD, and it was suggested that the termination factor Pcf11 functions through the CTD to relay mechanical force from the RNA to the

transcribing polymerase, causing a conformational change and eventual release of the polymerase (Zhang et al., 2005b).

Earlier work had shown that the association of the 8WG16 antibody with the CTD of stalled *D. melanogaster* RNAPII can trigger its release from the template *in vitro* (Zhang et al., 2004), further indicating that factors interacting with the CTD can affect the conformation of the polymerase complex.

A different model for termination of transcription and release of RNAPII from the template is the so-called torpedo model. According to this model, RNAPII release occurs when the polymerase is “torpedoed” by the yeast Rat1 RNA exonuclease, which degrades the nascent RNA from the 5’ end after cleavage of the primary transcript has taken place (Kim et al., 2004c). Studies in the human system identified the Rat1 homologue Xrn2 which, in conjunction with the Co-transcriptional cleavage (CoTC) mRNA sequence, effects transcription termination. The CoTC sequence is an autocatalytic RNA structure that undergoes rapid self-cleavage thereby creating an uncapped 5’ entry point for the Xrn2 exonuclease (West et al., 2004). The precise mechanism for release of RNAPII from the DNA template is unknown. However, the complexity of the protein apparatus responsible for transcriptional termination, pre-mRNA transcript cleavage and polyA-addition suggests that important discoveries are still to be made in this field of research (reviewed by (Bentley, 2005))

### **1.17 Premise for the work presented**

The association of Mediator with RNAPII, and its function in transcription, depends crucially on the CTD. Both the Kornberg Mediator and the Young Srb-Mediator is able to bind an unphosphorylated GST-CTD fusion protein *in vitro* (Myers et al., 1998; Thompson et al., 1993; Wilson et al., 1996), and the Kornberg Mediator can be displaced from RNAPII using the monoclonal antibody 8WG16, which specifically recognises the CTD repeat (Kim et al., 1994). As described above, work from several laboratories implicated hyperphosphorylation of the CTD in transcription initiation and promoter clearance, and the suggestion was made that hyperphosphorylation of the CTD during or before promoter clearance would lead to displacement of the Mediator complex either by a change in charge or conformation in holoRNAPII, or possibly as a consequence of competition for binding by factors that recognise phosphorylation patterns on the CTD (Svejstrup et al., 1997). Relevant competing candidates could be factors that have a role in signalling the start of initiation such as members of the RNA capping machinery (Pei et al., 2001) or other chromatin associated factors that are known to bind the phosphorylated CTD. Known CTD interacting factors include the proline isomerase Ess1 (Myers et al., 2001) and the Elongator complex (Fichtner et al., 2002; Otero et al., 1999; Svejstrup et al., 1997). Moreover, the selective binding of hypophosphorylated CTD peptide to immobilised TBP (Usheva et al., 1992) lends support to a model in which hyperphosphorylation of RNAPII in the PIC loosens interactions with PIC components (such as TBP and Mediator) and affect release of RNAPII into the coding sequence during promoter clearance. Indeed, RNAPIIA is known to preferentially associate with the preinitiation complex, and promoter clearance is correlated with CTD hyperphosphorylation. These conclusions were derived from experiments performed in the context of the multi-protein preinitiation complex, in which multiple different factors assemble and interact on a DNA template. In contrast, the intrinsic stability of the holoRNAPII complex in relation to CTD hyperphosphorylation has not been rigorously tested.

The first part of the present thesis describes my efforts in testing the effect of CTD hyperphosphorylation on the stability of the interactions between Mediator and RNAPII in the context of highly purified holoRNAPII complex *in vitro*. This work has entailed purification of multiple factors, and therefore also led to the discovery and initial

characterization of unexpected mechanisms that may have implications for the transcription cycle.

A majority of reports on the dynamics of CTD conformation have concluded that it is a very mobile, largely structure-less domain. At the same time, the CTD serves a conserved function as a coordinating platform for binding of multiple conserved factors during the transcription cycle. The interactions of some CTD binding proteins are mutually exclusive, but others seem to interact somehow simultaneously with RNAPII during the transcription cycle. Various CTD binding factors have been suggested to bring about structural features in the CTD by induced fit. The simultaneous binding of multiple factors may thus cause a general change in CTD conformation. CTD modification, such as phosphorylation or isomerisation of Ser-Pro linkages, may also elicit a change in CTD dynamics. A model was proposed whereby the unphosphorylated CTD attains a condensed spiral structure, whereas specifically Ser5 phosphorylation would cause disruption of the spiral (Meinhart and Cramer, 2004).

Elucidation of changes in CTD conformational dynamics in solution might shed light on the mode of binding of CTD interacting factors. The second part of the thesis mainly concerns the set-up of a system to measure the distance from the beginning to end of the CTD using Fluorescence Resonance Energy Transfer (FRET) under conditions of hyper- or hypo phosphorylation. The effect of CTD binding proteins on the CTD overall conformation will also be studied using this system.



## **2. Materials & Methods**

In this section, general procedures are explained before issues that are more specific are described. Accordingly, general DNA manipulation procedures precede descriptions of cloning strategies, and general protein purification procedures precede descriptions of the specific purifications performed.

### **2.1 DNA manipulation**

#### **2.1.1 Restriction digestion**

DNA to be digested was suspended in volumes of 10 $\mu$ l to 50  $\mu$ l of the relevant restriction buffer and digested with at least a 10-fold excess of restriction enzyme activity for 2 – 16 hours at the temperature recommended by the manufacturer. Restriction enzymes were from Promega and New England Biolabs (NEB)

#### **2.1.2 Ligation**

Ligations using NEB T4 DNA ligase (NEB cat. # M0202S) were normally done in a volume of 10 $\mu$ l to 20 $\mu$ l, in restriction enzyme buffer supplemented with 1mM DTT and (where specified) 1 mM hexamine cobalt chloride (Rusche and Howard-Flanders, 1985).

Ligations were done overnight at 16 degrees or in a temperature cycler using cycles of 16°C to 30°C for 10 sec each (Lund et al., 1996).

#### **2.1.3 PCR**

For purposes of *E. coli* colony-PCR to probe for presence and orientation of inserts (las-Yang et al., 1998), Taq polymerase was used (Roche, cat. # 11 146 165 001). The reaction buffer for Taq polymerase was [10 mM Tris-HCl pH=8.3 at 20°C, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl]. A 10 x stock was used when preparing reactions.

PCR reactions to produce inserts for purposes of cloning or and yeast genomic tagging were performed using proof-reading polymerases such as PFU DNA polymerase (Stratagene cat. # 600153) or KOD Hot Start DNA Polymerase (Novagen cat. # 71086). The reaction buffer for proofreading polymerases was the one supplied with the polymerase in question.

## Materials & Methods: DNA manipulation

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For plasmid templates, primers were typically used at a concentration of 1 pmol/ $\mu$ l reaction mix. For PCR from genomic templates, primers were used at a concentration of 0.3 pmol/ $\mu$ l reaction mix. PCR primer pairs are listed in the primer table appendix.

The program for temperature cycling was optimised for the individual reactions.

### **2.1.4 Sequencing**

Sequencing reactions were set up using the Big dye Terminator sequencing kit (ABI) and buffers as per manufacturer instruction. For plasmid sequencing, 150 ng of template and 3.2 pmol primer were used per reaction. Thermo cycling [Denaturation: 96 °C 10 sec, Annealing: Melting temp. of primer 5 sec. Extension: 60 °C 4 min] was done using a temperature cycler (MJ Research)

The products of sequencing reactions were purified using DyeEx spin columns (Qiagen cat. # 63204) dried in a vacuum (Speed-vac, Savant) and sent to the sequencing department of CR-UK at Lincoln's Inn Fields in London for processing. Sequencing was performed using a capillary sequencing machine (ABI Prism 3730).

### **2.1.5 Cloning of bacterial constructs for FRET assay**

The overall strategy was to amplify by PCR the different versions of eGFP as well as the CTD sequence and fuse them by ligation, before they were introduced into an entry vector in the Gateway system (Invitrogen). From this point onwards, they could be conveniently tagged with a selection of affinity tags available in the Gateway destination vector collection. The original *Aequorea* GFP protein has been optimised through directed selection of mutants and specific point mutations have shortened the time it takes for GFP to autocatalyse a rearrangement (known as maturation) of the residues surrounding the active site (Tsien, 1998). Other versions show altered fluorescence characteristics such as enhanced yellow fluorescent protein (eYFP), and enhanced cyan fluorescent protein (eCFP) (reviewed in (Tsien, 1998)). Fortunately, the extreme C- and N-term ends are identical and thus the same set of primers (primers 3 & 4, see appendix) could be used for amplification of different eGFP-derived sequences (called AFP for *Aequorea* fluorescent protein hereafter). Fusions of the CTD sequence with eGFP, eCFP and eYFP were all cloned in parallel and the procedure used was identical.

## Materials & Methods: DNA manipulation

Figure 3 illustrates the cloning strategy for the AFP-CTD insert using two-step PCR. The AFP and CTD reading frames were amplified by PCR in separate reactions using primer pairs 3 & 4 and 5 & 6 (see Materials & Methods) respectively. The products were purified on spin columns (Qiagen) and restriction digested (as above) using the PstI enzyme (NEB) to cut a site encoded in the 5' end of primers 4 and 5. Ligation of the resulting fragments created a fusion of AFP and CTD and this was amplified using primers 7 & 8. The 5'-end of primer 7 had the sequence CACC, which makes it compatible with insertion in an entry vector in the Gateway cloning system (Invitrogen). The CTD sequence was amplified from yeast genomic DNA using primers 9 & 10 and cloned into the Gateway pCR-BLUNT-II-TOPO vector (Invitrogen, cat. no K2800-20). This vector was sequenced to ensure correct sequence and used as a template for PCR using primers 8 & 9.

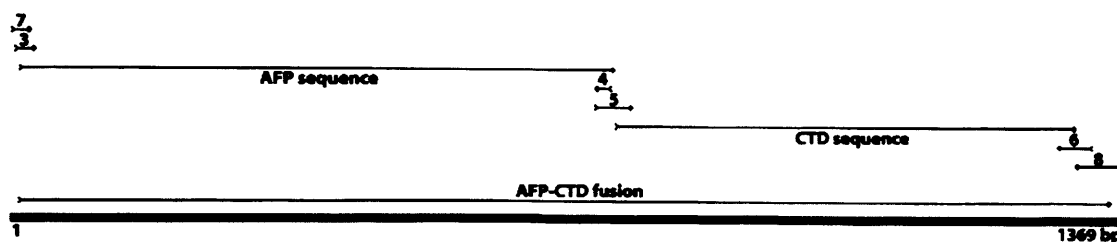
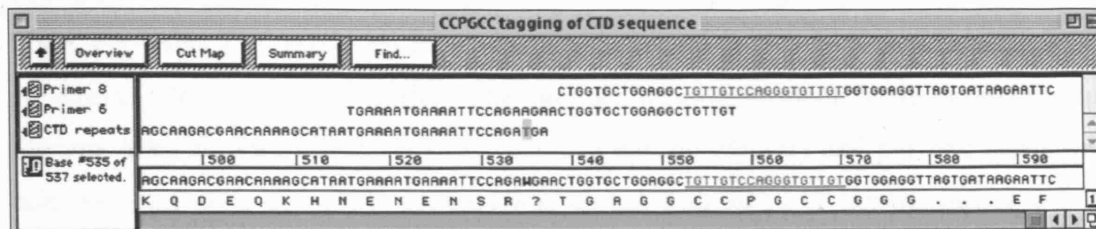


Figure 3 Cloning strategy for the AFP-CTD-CCPGCC construct

*The drawing shows the overall strategy for tagging the CTD sequence with a tetra-cysteine tag. The numbered arrows represent primers. The sequences of the primers can be found in the primer appendix.*

The two PCR products were purified on Qiagen PCR purification spin columns and eluted in T4 DNA ligase buffer (NEB). The products were mixed in equimolar proportions in a 20 µl reaction with 1 µl T4 DNA ligase and incubated overnight at 16 °C. The ligated fragments were used as template for PCR using primers 7 & 8 and the product was purified as above and cloned into the Gateway entry vector pENTR/SD/D-TOPO (Invitrogen cat. no K2420-20). The correct orientation of the insert was confirmed by colony-PCR and finally the insert was cloned by recombination into the bacterial Gateway destination vector pDEST15 (Invitrogen cat. no. 11802-014). This vector encodes a GST tag which is fused N-terminally to the protein of interest. The amino acid sequence context of the tetra cysteine tag is illustrated in Figure 4. The context of the tetra cysteine tag (**AGGCCPGCCGGG**) is identical to the context used in the Gateway destination vectors for in-cell labelling of CCPGCC tags (see the vector



An illustration from the program Sequencher (Gene codes Corp.) used to design the construct. Shown is an alignment of the C-terminal end of the CTD sequence ("CTD repeats"), and sequences of primers no. 6 & 8 that were used to tag the CTD. The consensus sequence shows the nucleotides encoding the tetra-cysteine tag in red. The bottom line shows the in-frame translation of the resulting protein. The "?" in the protein sequence designates where the endogenous TGA stop codon was changed to AGA (encoding arginine) to enable the tagging. Following the tag, a three-glycine spacer separates the CCCPGCC tag from three different in-frame stop-codons (marked ".") to ensure efficient termination.

### 2.1.6 Cloning of control constructs for FRET assay

### 2.1.7 Tetra-cysteine labelled calmodulin

The Cys6,7,10,11-calmodulin reading frame was amplified by PCR from the Tsien lab plasmid using primers #11 and #12. The PCR product was purified on spin column (Qiagen), and cloned into the Gateway entry vector (pENTR-SD/D-TOPO). Correct orientation was checked by colony-PCR spanning the backbone-insert interface and plasmid DNA was isolated from a correct clone using a mini-prep kit (Qiagen). Correct sequence was verified by sequencing. Finally the Cys6,7,10,11-calmodulin open reading frame was recombined into the destination vector pET-DEST-42 using the Gateway system LR enzyme mix, according to the manufacturer's instructions (Invitrogen). The pET-DEST42 bacterial vector fuses the protein of interest to a C-terminal nickel chelating tag which was used to purify the protein.

### 2.1.8 Cys6,7,10,11-calmodulin-CFP fusion

To fuse Cys6,7,10,11-calmodulin to the ORF of CFP the pENTR-Cys6,7,10,11-calmodulin Gateway entry vector created for His tagging Cys6,7,10,11-calmodulin (see above) was digested with AscI [20  $\mu$ l reaction containing 5 $\mu$ g DNA, 25 units AscI (NEB), 1x NEB restriction buffer 4, incubate 37°C, 3 hours] to open the backbone just after the Cys6,7,10,11-calmodulin ORF. The AscI enzyme was inactivated by heat treatment [70°C, 10 min] and then the AscI 5' overhang was filled in by adding 1/9

## Materials & Methods: DNA manipulation

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volume of 10 x BSA/dNTP's (0.5 mg/ml acetylated BSA/1 mM dNTP's) and 1µl T4 DNA polymerase [incubation 12°C, 30 min] and purified using spin columns (Qiagen PCR purification kit) and eluted (30µl 10mM Tris-HCl pH=8).

The CFP insert was made by PCR (using primers #3 & #4) with proofreading KOD polymerase in proprietary buffer. The product was dephosphorylated by adding 1µl Shrimp Alkaline Phosphatase (SAP, Promega cat. no M8201) and purified using spin columns as above. The PCR product was eluted with 30 µl of BamHI buffer and 20 units of BamHI was added and the reaction was incubated at 37 °C for 3 hours to cut the BamHI site encoded on primer #3. To blunt the staggered cut 1 µl of Mung Bean nuclease was added and the reaction was incubated for a further 30 min at 30°C. Lastly, the product was purified using spin columns as above and eluted in 30µl elution buffer [10 mM Tris-HCl, pH 8.0].

The plasmid and the insert could now be ligated to fuse the 3' end of the Cys6,7,10,11-calmodulin ORF in-frame with the CFP ORF. Ligation was performed as described above and the product was used to transform *E. coli* SHURE cells (Stratagene cat. no. 200238) strain by heat shock according to manufacturer instruction and plated on LB plates containing 100 µg/ml Ampicillin to select for successful transformants. Colonies were screened for orientation of insert by colony PCR using primers (#11 and #4), clones with correct orientation were mini prepped (Qiagen miniprep kit), and correct sequence was verified by automated sequencing as described above.

## 2.2 Yeast manipulation

### 2.2.1 Epitope tagging in yeast

Tagging of the 3' end of yeast genes in their chromosomal location was accomplished by transformation of a PCR product made by amplification from a vector library obtained from ((Knop et al., 1999) and unpublished). To stimulate recombination, pairs of PCR oligo's each contained 50 bp of homologous sequence from either side of the stop-codon of the gene of interest (Primers 1 and 2, - see Materials & Methods) The template vector sequence encoded the affinity tag followed by a stop codon and a heterologous trophic selection maker (*K. lactis* *TRP1* or *URA3* or *S. pombe* *HIS3*). Using a heterologous marker reduced the risk of mis-targeting through recombination with homologous sequence elements of endogenous mutated marker gene alleles in the *S. cerevisiae* genome. Protein tagging sequences employed were a triple influenza

## Materials & Methods: Yeast manipulation

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haemagglutinin (HA) epitope which is recognized by the mouse monoclonal antibody 12CA5 raised against the peptide sequence YPYDVPDYA (Field et al., 1988). The *K. lactis URA3* gene (genbank accession Y00454) comprises 1167 bp. This, in addition to the 50 bp homologous sequences on the oligo's, as well as the epitope-tag encoding sequence, results in a expected PCR product of 1400pb for 3HA::URA tagging PCR product. The PCR was set up as described above, using 4% DMSO final and processed using the following program. Initial denaturing: 95°C 3 min, then five cycles of [95°C 20 sec/55°C 20 sec/72 °C 3 min], 25 cycles of [95°C 30 sec/Gradient 55°-70°C c 30 sec/72 °C 3 min] and lastly 72°C 10 min to finalize products.

### **2.2.2 Mating yeast strains**

To construct a yeast strain devoid of Elongator subunits, yeast W303-based strains # 7 & #8 (see strains table) were mated by cross-streaking on a selective minimal plate lacking adenine, leucine and tryptophan. Only the offspring of this cross was able to grow on the selective plate. The resulting diploid strain was induced to sporulate on sporulation plates and 10 tetrads were dissected on YPD and replica-plated to selective media lacking relevant trophic markers, and haploid progeny cells that grew under triple selection were isolated.

### **2.2.3 Yeast transformation**

Yeast cells were transformed with either the Lithium Acetate DNA precipitation method or by electroporation.

#### **2.2.4 Transformation using Lithium acetate precipitation**

This procedure is according to (Gietz and Woods, 2001). YPD cultures of 50ml were inoculated and grown overnight at 30°C, so that they would reach a density of  $5 \times 10^7$  cells/ml at harvest time. Next day, the culture density was checked using a cell counter (Beckman Coulter model Z2) and competent cells were pelleted at 3000 rpm 5 min (Beckmann GH3.8 rotor) and resuspended in 50 ml sterile H<sub>2</sub>O. This step was repeated once. For each transformation,  $2 \times 10^8$  cells were pelleted in a 1.5 ml Eppendorf tube in a microcentrifuge at high speed ( $>10$ k rpm, 30 sec, Eppendorf 1015) and the supernatant was discarded. Before transformation, an aliquot of 2 mg/ml single stranded salmon sperm DNA (Sigma D-1625) was boiled (95°C, 5 min) and rapidly cooled in wet ice.

All other reagents were sterilised by filtering through a 0.2 µm syringe filter (Schleicher & Schuler) before use.

To each yeast pellet, the following reagents were added in sequence:

240 µl Polyethylene glycol (PEG) 3500  
36 µl 1M Lithium Acetate  
50 µl Single-stranded Salmon Sperm DNA (freshly vortexed)  
34 µl Plamid DNA in H<sub>2</sub>O (1 -5 µg DNA)

The contents were now thoroughly mixed by vigorous vortexing. The tubes were incubated at 42 °C for 30 min and then briefly pelleted at high speed. The transformation mixture was removed and discarded and the pellet was resuspended in sterile H<sub>2</sub>O. A volume of 10µl – 100 µl of the resuspended cells was plated on appropriate selective minimal plates and incubated at 30°C for 3 – 4 days.

### 2.2.5 Electroporation

Cells grown to stationary phase were used for electroporation according to (Grey and Brendel, 1992) using a Biorad Gene pulser with 0.1 mm cuvettes (Equibio) at settings of (0.55 kV, 25 µF, 600Ω, time constant of 7-9 ms). 50 ml cells at a density of  $1.6 \times 10^7$  cells/ml were harvested by centrifugation (3200 rpm, 2 min, 4°C, Beckmann GH3.8 rotor). Cells were washed in sterile H<sub>2</sub>O, re-pelleted as above, and re-suspended in 2 ml 1M sorbitol, re-pelleted, and gently resuspended in 1 ml [20 mM Hepes, 1M sorbitol]. For each transformation, 100µl cells were mixed with 2µl PCR product and electroporated in a 0.1 mm single-use sterile cuvette. Immediately after the pulse, cells were gently resuspended in 1 ml 1M Sorbitol and plated on appropriate selective minimal plates.

### 2.2.6 Tagging RNAPII with c-myc and tetra-cysteine motif

A PCR product was made with primers encoding the tag and a heterologous *K. lactis* *URA3* marker. The strategy is illustrated in Figure 5

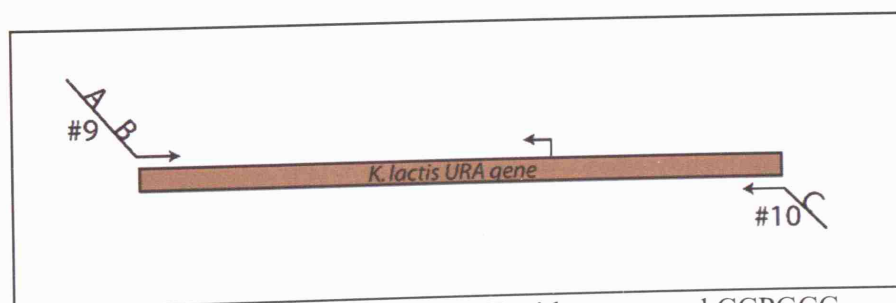


Figure 5 Strategy for tagging RNAPII with c-myc and CCPGCC

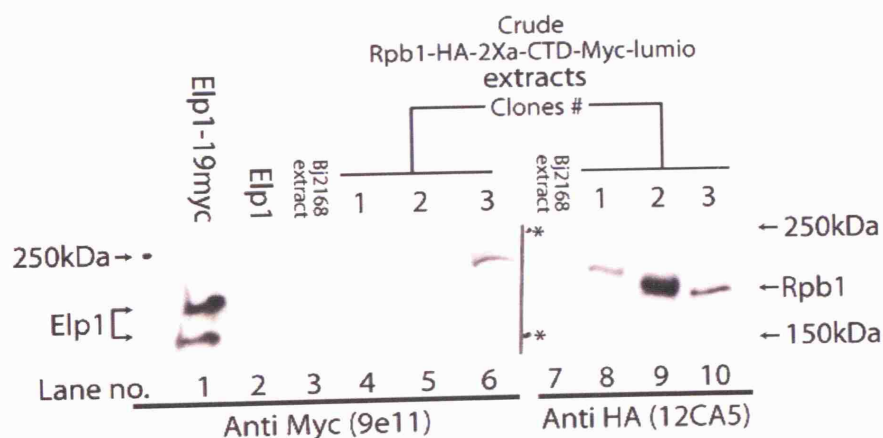
## Materials & Methods: Yeast manipulation

The primers #9 and #10 were used to amplify the *Klactis URA3* gene from a vector obtained from the Nasmyth lab. For correct targeting of recombination sections A and C of primers #9 and #10 respectively are 50 basepair regions that combined are identical to the 100 basepairs surrounding the *Rpb1* stop codon. Section B of primer #9 encodes an in-frame c-myc and tetra-cysteine tag as well as three in-frame stop codons. The heterologous *URA3* gene serves as a trophic marker to select for transformants.

Recombination served to replace the C-terminal stop-codon of the plasmid-borne RNAPII with the combined c-myc/tetra-cysteine tag.

### 2.2.7 Verification of the presence of tags on Rpb1

After selection on –URA plates, three transformants were streaked to single colonies and used to produce extracts, which were immunoblotted for the presence of HA and myc epitopes. The blot is shown in Figure 6. Lane 1 & 7 was loaded with a positive control for the c-myc antibody (purified 19-myc tagged Elongator complex from Cathy Greenwood in the lab). The doublet probably arises through degradation of the 19 myc tag. Negative blotting control was crude extract from the untagged BJ2168 strain and purified Elongator without the HA tag. As can be seen only clone three displays signals in both blots. The 5% SDS-PAGE gel has slipped slightly during mounting in the transfer tank causing warping of the bands, but both HA and c-myc signals are at the level of Rpb1 indicating that only in the case of clone three, did recombination find the correct target on the episomal vector.



**Figure 6 Verification of the presence of tags on Rpb1**

A 5% SDS-page gel was loaded with the crude extracts from three different clones (lanes 4-6 and 8-10) and a BJ2168 yeast extract negative control for specificity (lanes 3 + 7), purified Elp1-HisHa negative control (lane 2) and a Elp1-19myc extract as a positive control for the 9e11 antibody (lane 1). The doublet in lane 1 is caused by degradation in this prep of 19myc tagged Elp1 (Catherine Greenwood – personal communication). The asterices indicates the position of the markers indicated by arrows on the right. The line between lanes 6 & 7 indicates the separation between two membrane halves. Clone #3 has both the HA tag and the 19myc tag on a band the size of Rpb1.



Clone 3 was grown in large-scale fermentation (100 litre) in YPD and cell were harvested and protein purified as described below. The final verification of the presence of the tags on the purified protein can be found in the Results section.

### **2.3 Polyacrylamide gel electrophoresis**

A 30% Acrylamide/Bis-acrylamide solution (ratio 37.5:1, Biorad cat. no 161-0157) was used to cast SDS-PAGE gels. Stacking gels contained 4% (volume:volume) polyacrylamide and separating gels were from 5% - 15% (volume:volume) polyacrylamide. Gel buffers were made in stock concentrations and kept at RT.

#### **4x Stacking Buffer:**

0.5 M Tris-Cl, pH 6.8  
0.4% SDS

#### **4x Resolving Buffer:**

1.5 M Tris-Cl, pH 8.8  
0.4% SDS

#### **5 x SDS-PAGE sample buffer**

Sample buffer contained [300mM Tris-Cl pH 6.8, 60% glycerol (volume:volume), 4% SDS (w:v) and 10%  $\beta$ -mercaptoethanol (volume:volume), 0.04 % bromophenol blue (w:volume)]

Protein samples were cleared by centrifugation (14k rpm, 20 sec) mixed with sample buffer and incubated at 95°C for 3 – 5 min before loading on the gel. Gels were run at a constant voltage depending on the dimensions of the particular gel until the bromophenol dye front reached the end of the gel or, in the case of 5% SDS-PAGE gels to detect mobility shifts of phosphorylated Rpb1, until the pre-stained 175 kDa marker band (New England Biolabs cat. on# P7708S) had just run off, the edge of the gel.

#### **2.3.1 Sample concentration using TCA precipitation**

Sample volumes were supplemented with 1/20 (volume/volume) of 2 mg/ml Deoxycholic Acid (Sigma cat. no. D2510) and 1/9 (volume/volume) of 100% Trichloroacetic acid (Sigma cat. no. 522082). Mixed, incubated on ice 30 min and centrifuged 45 min at 4°C before removing supernatant from pelleted protein. Pellet was washed with a volume of ice cold acetone corresponding to the original protein sample and after removal of supernatant the sample was dried briefly in a speedvac (Savant) and resuspended in 15  $\mu$ l 1xPAGE sample buffer.

#### **2.3.2 Concentration using StrataClean resin**

Strataclean resin (Statagene cat. # 400714) is a hydroxylated silica based resin, which according to manufacturer information interacts with proteins in a phenol-like fashion. The resin was used to concentrate protein samples prior to SDS-PAGE analysis. Usually 5 -10  $\mu$ l of resin suspension was added directly to the protein solution and samples were mixed vigorously for 1 min, and left to stand at RT 1 min. The resin was pelleted (6000 rpm, Eppendorf 5415 centrifuge), the supernatant removed, and finally the pellet was resuspended in 1 x SDS-PAGE sample buffer, heated to 95°C 3 min, and loaded on SDS-PAGE.

## 2.4 Immunoblotting

### 2.4.1 *Transfer conditions*

Buffer for blotting was made from a 10 x stock (30g Tris-Base, 145g glycine pr litre) in cold MilliQ H<sub>2</sub>O. An ice pack was inserted inside the transfer tank and to cool the buffer further during transfer, the whole transfer tank itself was placed in a tray, and ice was packed around it. The set-up was placed on a magnetic stirrer and a small stirrer-bar was used to circulate the buffer during transfer.

### 2.4.2 *Equilibration of gel and membrane*

Nitrocellulose membranes were equilibrated directly in transfer buffer for 10 min. When using PVDF membrane (Immobilon P, Millipore) this was cut to size and initially immersed for 30 sec in 100% Methanol to wet the surface, then transferred to H<sub>2</sub>O (MilliQ quality) for 2 minutes to hydrate the membrane. Finally, the membrane was equilibrated for 10 min in transfer buffer. The gel was also equilibrated in transfer buffer for 10 min before assembly of the transfer sandwich. To avoid bubbles, the transfer sandwich was assembled under a layer of transfer buffer in the following way: [Anode/Fiber pad/3x3MM filter paper/gel/membrane/3x3MM filter paper/Fiber Pad/cathode] and transferred to the blotting tank. The transfer-sandwich was immersed in the wet transfer tank and proteins were transferred at 100 V constant for 90 min (Bio-Rad, XT gel system) or 100 V constant for 60 min in the case of mini gels (protean3, Bio-Rad). Larger gels were blotted in a similar manner, using a Bio-Rad Transblot cell at 0.5 Amps constant for 2 hours.

After blotting, the sandwich was taken apart from the membrane side and the outline of the gel corners was drawn on the back of the membrane using a felt pen. When using

nitrocellulose membranes, transfer efficiency was checked by Ponceau staining (see below). For PVDF membranes, the membrane was immersed into 100% methanol for 30 seconds and dried for at least 30 min on filter paper. This procedure is described in the manual for the Immobilon-P membrane (Millipore cat #.IPVH 000 10). The dry PVDF membrane is very hydrophobic and only the proteins bound on its surface will interact with hydrophilic solvent. The drying step therefore effectively constitutes a blocking step and further blocking through milk is not needed. After this, the trans-illumination method (see below) was used to check for successful transfer of proteins.

### ***Transfer verification procedures***

#### **Ponceau S staining**

Transfer efficiency was ascertained by reversible staining with Ponceau S when using nitrocellulose membranes (Hybond-C, Amersham cat. # RPN303E). Directly after transfer, the gel-membrane sandwich was taken apart and the blot was immersed for a few minutes in a solution of 0.1% PonceauS (w/v) in 5% (v/v) acetic acid (Sigma cat no. P7170-1L) and then destained in several washes of H<sub>2</sub>O until clear bands appeared. Subsequently, the bound Ponceau S was removed completely by incubation of the membrane in blocking buffer (5% low-fat dehydrated milk in PBS, 0.05% Tween 20).

### ***2.4.3 Trans-illumination***

Immersion of the dried membrane in 20% methanol results in partial re-hydration of the protein containing areas of the membrane resulting in translucence here, whereas other areas stay opaque. Protein bands are then visualised by trans-illumination using a light box.

### ***2.4.4 Blocking***

PVDF membrane was blocked using the quick blocking procedure according to manufacturer instruction. To convert the membrane to its hydrophobic state, it was immersed in 100% methanol for 30 seconds and dried on filter paper for 20-30 min.

Nitrocellulose membranes were first stained with Ponceau S and then blocked in blocking buffer for 1 hour to overnight

#### **2.4.5 Antibody incubation**

Antibody solutions were made up in blocking buffer consisting of phosphate buffered saline (PBS) with 5% non-fat dry milk (weight:volume) and 0.035 % Tween-20. The percentage of Tween-20 is critical because higher concentrations make the dried PVDF membranes (see above) re-wet and noise from the now hydrophilic membrane starts to be a problem. The rabbit polyclonal Med1, Med4, Fcp1 and Ccl1 antibodies were diluted in blocking buffer in ratios (volume:volume) of 1:10000, 1:5000, 1:500, and 1:333, respectively. The mouse monoclonal antibodies 4H8 and 8GW16 were used at dilutions of 1:1000. For detection of the protein A part of the TAP tag, the soluble peroxidase anti-peroxidase complex (Sigma cat. # P1291) was used at a dilution of 1:2000.

#### **2.4.6 Detection**

The membranes to be blotted were cut in strips according to pre-stained molecular weight markers (Bio-Rad cat # 161-0373EDU) loaded on either side of the gel. The individual strips were rolled up on the inside of 50 ml tubes and a relevant antibody solution was added. The tube was incubated on a roller either 1 hour at RT or overnight at 4°C. Membranes were washed on a rocking table using three changes of 200 ml PBS each, over 20 min. Secondary antibodies directly linked to horseradish peroxidase and were diluted in blocking buffer at a ratio of 1:10000(volume:volume). Secondary antibodies were: sheep anti-mouse (cat. # NA931-1ML) and donkey anti-rabbit-HRP (cat. no#. NA934-1ML), both from GE Healthcare. Membranes were incubated on a rocking table for 30 min at RT and then washed as above. Chemo-luminescence detection was done using the SuperSignal West Pico Substrate from Pierce (cat. # 34076). Membranes were placed protein-side-up and covered in the ECL substrate. After 5 min incubation the edge of the membrane, strips were blotted with tissue to remove excess substrate and the strips were mounted onto plastic foil protein-side-down. The membrane was turned and placed in a cassette and film was exposed.

## **2.5 Bacterial protein expression**

### ***2.5.1 Growth and induction of bacterial cultures***

For protein expression using isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) induction, growth medium was either 2xYT or LB supplemented with the relevant antibiotics. Ampicillin was used at a concentration of 100  $\mu$ g/ml, chloramphenicol was used at 50  $\mu$ g/ml and kanamycin was used at 50  $\mu$ g/ml. For overnight inoculation, culture was set up the previous day and the main culture was inoculated at a maximal dilution of 1:100. In the following, the particular parameters for recombinant protein growth and induction varied according to the protein in question.

Cells were grown in an incubator shaking at 200-250 rpm at a temperature of either 18°C, 22°C, 25°C, 30°C, or 37°C. The growth of the culture was followed by reading its optical density at 600 nm ( $OD_{600}$ ), and at the  $OD_{600}$  required, addition of between 0.1 mM and 1 mM IPTG was used to induce transgene expression. Induction was done between 4 and 12 hours, and cells were harvested by centrifugation at 6000 rpm 10 min at 4°C. The lysis buffer was made 2x and supplemented with 1x protease inhibitors and 1-5 mM reducing agent Dithiothreitol (DTT),  $\beta$ -mercaptoethanol ( $\beta$ -Me) or tris(2-carboxyethyl)phosphine (TCEP). Wet cell weight was measured and cells were resuspended in same amount (volume/weight) of ice cold 2xlysis buffer. Finally, 1x lysis buffer was added to a final volume of between 1/50 and 1/100 of the original culture volume. Addition of 1  $\mu$ l Benzonase (Novagen) per 10 ml of crude lysate reduced the viscosity caused by DNA. Cells were disrupted by high pressure using a homogenizer machine (Stansted Fluid Power Ltd, Stansted, UK) at a pressure of above 1000 psi until the lysate had lost viscosity.

Lysate was cleared by centrifugation in a Sorvall SS34 rotor (15000 rpm, 30 min, 4°C) or ultracentrifugation using a Beckmann Ti45 rotor (42000rpm, 45 min, 4°C). The clarified lysate was removed using a plastic pipette.

## **2.6 Staining and labelling procedures**

### **Coomassie staining of SDS-PAGE analysis gels**

The gel to be stained was immersed in a plastic tray containing [0.5% (w/v) Brilliant Blue R250, 45% (v/v) ethanol and 10% (v/v) acetic acid] and heated using a microwave oven until almost boiling. The gel was left on a rocking table for 30 min – 1 hour and

then de-stained in [40% (v/v) ethanol, 7% (v/v) acetic acid] by re-heating in a microwave oven. When de-staining was complete, the gel was dried between 2 sheets of cellophane support, using a Bio-Rad GelAir gel drying system (Bio-Rad cat. # 165-1771).

### **2.6.1 Silver staining**

Staining was done in clean plastic containers. The gel to be stained (either after Coomassie staining or immediately after electrophoresis) was fixed in [50% Methanol, 12% Acetic acid and 0.5 ml/liter 37% HCOH (formaldehyde)] for at least 1 hour. The fixing solution was exchanged for 50% EtOH, by soaking for at least 20-30 min. The gel was sensitized by soaking in [0.2 g/litre Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>] for exactly 1 min whereafter the gel was washed 3 times in H<sub>2</sub>O (20 seconds each). The gel was placed in a staining bath of [2 g/l AgNO<sub>3</sub> (silver nitrate), 0.75 ml/l 37% HCOH] for 20-45 minutes depending on gel size/thickness and protein amount to be stained. After staining, the gel was washed 2 times in H<sub>2</sub>O, 30 sec each. Finally, the gel was developed by soaking in [60 g/liter Na<sub>2</sub>CO<sub>3</sub>, 4 mg/liter Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.5 ml/liter HCOH] until no more specific signal could be obtained. The developing process was stopped by shaking incubation with 1% Acetic acid for 1 hour and the gel was dried as above.

### **2.6.2 Labelling using Lumio Green**

In-gel labelling was done according to manufacturer instruction. Protein samples (crude or purified) were cleared by centrifugation and mixed in a ratio of 1:3 with the 4x loading buffer provided. To a reaction volume of 20 µl, 0.2 µl of the Lumio Green reagent (Invitrogen) was added and the sample was incubated at 70°C for 10 min. After briefly cooling to RT, 5 µl of Lumio enhancer was added. After a brief incubation, samples were loaded onto SDS-PAGE. After electrophoresis the gel was removed from the glass plates and documented in a Fuji Las—3000 gel documentation system set for Sybr green detection with an aperture of F 0.8. Gels handled this way could subsequently be Coomassie stained.

### **2.6.3 Lumio labelling in solution**

Protein to be labelled with the Lumio green reagent (Invitrogen) was concentrated to at least 3 mg/ml before labelling, using centrifugal filter units (Centricon Millipore). A high concentration of protein was found to be necessary for efficient labelling (not shown). The buffer was exchanged by dialysis or G25 size exclusion chromatography

into labelling buffer (50mM MOPS pH=7.4, 2 mM MES, 1mM TCEP, 1mM EDTA) and incubated 2 hours at 4 °C to ensure full reduction of cysteine groups before labelling. 1 µl of protein stock was then added to 39 µl of PBS, and 0.2 µl Lumio green (40 nM) or Lumio Red reagent was added for a final concentration of Lumio reagent of 0.2 nM final concentration. The protein was incubated at RT for 3 hours for labelling to proceed. The labelled protein was either analysed by SDS-PAGE followed by immediate visualisation in a LAS 3000 imaging system (Fujifilm) or the protein was purified by G25 chromatography and measured in a Varian Eclipse spectro-fluorometer (Varian GmbH).

## 2.7 Protein purification procedures

### 2.7.1 *Protease inhibitors*

Protease inhibitors were dissolved sequentially in absolute ethanol and typically kept at -20°C in a 100x stock concentration. Protease inhibitors were slowly added to buffers immediately before use. The final concentration of the individual constituents is listed below:

Benzamidine-HCL	1.9	mM
Phenylmethylsulfonyl fluoride (PMSF).	0.97	mM
Pepstatin A	2	µM
Leupeptin	0.523	µM

### 2.7.2 *Buffer preparation*

Buffers in this thesis are described by a letter identifying the formulation and a suffix denoting the salt concentration of the buffer (example: A<sub>0</sub> denotes buffer A with no salt). Buffers were prepared and brought to the temperature of use before checking and adjusting pH. Buffers for chromatography were prepared as 3x or 10x base buffers without salt and glycerol. For a gradient column run, the buffer with the highest salt concentration was first prepared adding salt and glycerol. The buffer of the lowest concentration of salt was prepared by diluting the high salt buffer in 1x base buffer containing glycerol. The dilution was either done manually or by the FPLC system during the run. Immediately before use, all buffers were degassed and 1/100 volume of a 100x protease inhibitor cocktail was added and the column was equilibrated in 0.5 -1 column volume of protease inhibitor containing buffer.

### ***2.7.3 Column packing***

The different column materials were either regenerated after use or dissolved from powder according to manufactures instruction. Hydrated column resin was degassed under vacuum at the temperature of operation (usually 4°C) for at least 20 min before pouring. Columns were gently poured into Bio-Rad column holders from 5 cm to .5 cm inner diameter (ID) taking care not to introduce bubbles. A pressure adaptor was fitted and the column was put under flow with equilibration buffer. Before loading protein, all columns were checked for equilibration. Equilibration of the column material was verified by checking that pH and conductivity readings of inflow vs. effluent were identical.

### ***2.7.4 Column regeneration***

Columns were washed in high salt after every run and brought to low salt conditions with H<sub>2</sub>O. Regeneration was done according to the instructions for the particular resin, which generally involved washing the 1-3 column volume of 0.5 M NaOH or KOH, then washing with H<sub>2</sub>O until the pH began to drop and finally re-equilibrated with base buffer without salt or glycerol. To preserve the column material from microbial/fungal attack sodium azide (NaN<sub>3</sub>) was added to a final concentration of 0,02%, and the column material was stored at 4°C in the buffer recommended by the manufacturer.

### ***2.7.5 Cell lysis and preparation of yeast extract***

Culture volumes less than 12 litres were grown in 6 litre shaking Erlenmeyer flasks (2 litre culture per flask) at 30°C. Cells were harvested by centrifugation at 6000 rpm, 15 min, 4 °C (Beckman SLC6000 rotor) washed in ice cold H<sub>2</sub>O, collected and re-pelleted as before. The volume of cells was measured by weighing (1 gram corresponding to 1 ml) and the cell pellet was resuspended in an equal volume of ice cold 3x lysis buffer to make a cell paste. The specific formulation of the lysis buffer depended on the protein to be purified. The yeast paste was extruded through a 50 ml syringe into an aluminium foil container fully immersed in liquid nitrogen to form so-called “popcorn” or “spaghetti”. The resulting snap-frozen yeast cell paste was stored at -80°C until the time of use. When needed, the yeast cells were disrupted by freezer milling (SPEX 6850 Freezer/Mill) under liquid nitrogen using 4 cycles of [2 min milling (at 20 impacts pr.



second)/2 min cooling]. The powder was thawed at 4°C and cell debris was cleared by ultracentrifugation at 32k rpm (Beckman Ti 45 rotor), 1 hour, 4°C.

Larger scale cultures (>50 litre) were inoculated and grown by Tina Scopa at the CR-UK fermentation department at Clare Hall. Tina also harvested and lysed cells according to my instructions. Yeast cells were grown in a batch fermentation vessel to mid-log phase or early stationary phase (depending on purification purpose) and harvested by continuous centrifugation. The volume of cells were measured by weighing (1 gram corresponding to 1 ml) and the cell paste was resuspended in ice cold 3x lysis buffer as described above. Cells were disrupted by cooled continuous bead beating at <12°C until disruption was judged to be maximal by microscopic examination (>70%). Cell lysate was cleared from debris by centrifugation at (14 k rpm, SLA1500 rotor, 30 min, 4 °C) and the supernatant was aliquoted into 50 ml tubes and snap- frozen in liquid N<sub>2</sub>. All lysates were stored at -80°C until the time of use.

### ***2.8 Protein fractionation by precipitation***

#### ***2.8.1 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation***

Freshly ground powdered solid NH<sub>4</sub>SO<sub>4</sub> was added slowly to the protein solution which was gently stirred with a stirrer bar at 4°C, taking care not to cause protein oxidation through frothing. The protein solution was left to equilibrate after NH<sub>4</sub>SO<sub>4</sub> addition from 40 min to 1 hour before centrifugation at >20.000 g using a SS34 rotor (Sorvall) or a Ti45 ultracentrifugation rotor (Beckman).

#### ***2.8.2 Polyethyleneimine precipitation of DNA/chromatin-associated proteins***

Concentrated Polyethyleneimine (PEI) (Supelco cat. # 21195-U) was diluted in MilliQ H<sub>2</sub>O and pH was adjusted to 7.9 with concentrated HCl. The solution was finally diluted to 10% and stored at room temperature. PEI precipitation was done at 4°C by slowly adding a 10% PEI solution to the crude protein extract with gentle stirring at 4°C until the final concentration was 0.25%. The mixture was left to equilibrate at least 1 hour and the pellet was recovered by centrifugation (14 rpm, 30 min, 4°C, Sorvall SLA1500 rotor). Washing was done by re-suspension of the pellet in low conductivity buffer (corresponding to 100 mM KOAc) by Dounce homogenisation at 4°C, until no clumps remained. The precipitate was re-pelleted as above and protein was eluted from the PEI by adding elution buffer of a higher conductivity (>400 mM KOAc) and resuspension as before.

## **2.9 Chromatography**

### **2.9.1 TAP tag purification**

The procedure followed was a modification of the protocol described (Puig et al., 2001). Correct assembly of multi-protein complexes depends critically on the constituent proteins being expressed in the cell to endogenous levels. Hence, rather than using over-expression of individual recombinant subunits, protein complexes were purified from yeast strains carrying genes modified at their chromosomal locus to incorporate the tandem affinity purification (TAP) tag at the C-terminal end. This tag fuses the calmodulin binding peptide (CBP) to the protein of interest, followed by a Tobacco Etch Virus (TEV) protease site that separates the CBP tag from a protein A domain. The protein A binds to the constant region of many antibodies of the IgG subclass and the CBP peptide binds tightly to Calmodulin in a  $\text{Ca}^{2+}$  dependent manner.

Purification is carried out entirely at 4°C by loading the starting protein on to IgG Sepharose 6 fast flow (Amersham Biosciences cat. # 17-0969-01). A bed volume of 200µl IgG for each 10 liter yeast culture was used. The IgG resin was prepared by washing in TST/0.5 M Acetic acid, as described under Preparation of IgG resin below. Finally, the resin was equilibrated in the same buffer as the extract lysis buffer (>10 column volume) and added to the crude extract, typically in 50 ml tubes. After binding for 2 hours on a roller-table at 4°C, the resin was transferred to a Poly-Prep (Bio-Rad) empty chromatography column and unbound protein was removed by washing extensively (>100 column volume) in IPP150 [10mM Tris-Cl pH=8.0, 150mM NaCl, 0.1% NP40, Protease inhibitor cocktail]. To elute bound protein, the column buffer was first exchanged by washing in 50 column volume TEV cleavage buffer [10mM Tris-Cl pH=8.0, 150mM NaCl, 0.1%NP40, 0.5mM EDTA, 1mM DTT]. A 2.5 column volume of TEV cleavage buffer containing (1-2 units/µl of TEV protease) was added and 1.5 column volume was allowed to pass before the flow was stopped with a stopper. The column was covered and left overnight at 4°C for proteolysis to take place while limiting protein oxidation. Protein was eluted in 1 column volume fractions by adding cleavage buffer. At this point, the elution fractions were sampled and 5-10 µl of each was analysed by SDS-PAGE and Coomassie or silver staining of proteins.

If further purification was deemed necessary, IgG elution fractions containing relevant proteins were pooled and diluted with three volumes of calmodulin binding buffer, [10mM Tris-Cl pH=8.0, 10mM β-mercaptoethanol, 150mM NaCl, 1mM Mg-acetate,

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1mM Imidazole, 2mM CaCl<sub>2</sub>, 0.1% NP40, 1x protease inhibitor cocktail]. For each ml of IgG eluate 3 µl 1M CaCl<sub>2</sub> was added. Proteins were loaded onto a poly-prep (Bio-Rad) column containing 200 µl bed volume of Calmodulin Sepharose 4B (Amersham Biosciences cat. # 17-0529-01), previously equilibrated in IPP150. Protein was left to bind for 2 hours at 4°C on a roller table. Unbound protein was removed by washing extensively (>100 column volume) with IPP150 and proteins were eluted in fractions of 1 column volume by addition of Calmodulin elution buffer [10mM Tris-Cl pH=8.0, 10mM β-mercaptoethanol, 150mM NaCl, 1mM Mg-acetate, 1mM Imidazole, 2mM EGTA, 0.1% NP40, 1x protease inhibitor cocktail] to strip the calcium ions off the column causing the Calmodulin to release the bound calmodulin binding peptide. Elution fractions were analysed by SDS-PAGE and Coomassie or silver staining of proteins.

Tandem affinity purification of TAP tagged Mediator was carried out following the procedure published (Puig et al., 2001; Rigaut et al., 1999). In the case of Mediator purification from a Med14-TAP expressing strain (Borggrefe et al., 2001), the procedure was slightly different. To capture the complex from crude whole cell extract, purification on Heparin-sepharose was the initial step in the procedure, a modification devised in the Gustafson lab at the Karolinska Institute in Sweden (personal communication). After this step, the TAP tag procedure just described was followed. Heparin chromatography is described below.

### **2.9.2 Metal chelating affinity purification**

Resin for metal chelating affinity purification was purchased from Qiagen (Ni-NTA Superflow cat. no.30430). Buffers for metal chelating affinity purification were devoid of EDTA to avoid stripping the column of metal ions. Addition of DTT was also avoided. They generally contained a buffer system (Tris-Cl, Hepes-Cl or a phosphate buffer) and at least 300mM KCl (or KOAc) to avoid unspecific ionic interactions, and also contained a minimum of 10 mM or 20 mM imidazole to out-compete weak, unspecific interactions with the resin. Protease inhibitors were added to buffers just before use. The resin was equilibrated in starting buffer formulated to be similar to the sample buffer with the addition of salt and imidazole. Imidazole was added from a 1 M stock solution with a pH of 7.5. Buffers for the purification from very crude sources also contained 0.3 % TritonX-100 to solubilise and extract membrane-trapped proteins, and to diminish unspecific hydrophobic interactions. Binding of protein was done either

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in batch for 2-3 hours with gentle agitation at 4°C or by gravity flow through Ni-NTA resin packed into a Bio-Rad cartridge (Poly-Prep or EconoPac) and pre-equilibrated with 5 column volume of starting buffer. In the case of batch binding, the protein-laden resin was gently resuspended and poured into a column cartridge. Re-circulation of the effluent was used until every resin bead was collected. The column was washed extensively by gravity flow (>50-100 column volume). In some cases, (purification of GST-CFP-CTD-Lumio), wash buffers contained 150 mM imidazole to overcome non-specific binding of contaminating bands. Finally, bound proteins were eluted in 1 column volume batches with buffer containing at least 250 mM Imidazole. Eluted proteins were generally dialysed or buffer exchanged by size exclusion chromatography columns (PD10, GE biosciences) to remove the imidazole and raise the glycerol concentration of the storage buffer before snap freezing in liquid nitrogen. Purified proteins were kept at -80 °C.

### 2.9.3 Glutathione-S-transferase affinity purifications

#### 2.9.4 Purification of GST-Ess1

A plasmid expressing Ess1-GST was obtained (a kind gift from the Greenleaf lab (Morris et al., 1999)) and transformed by heat-shock into *E. coli* BL21(DE3) plysS cells. Throughout this purification, selective medium and plates contained 100 µg/ml Ampicilin, and 50 µg/ml chloramphenicol. Cells were plated on selective LB plates and colonies were isolated and re-streaked to single colonies. For protein expression, one colony was used to inoculate 15 ml of LB containing antibiotics and the starter culture was grown until turbid and then used to inoculate (at a ratio of 1:100 volume:volume) three Erlenmeyer flasks, each containing 400 ml 2xYT medium (with antibiotics). The culture was incubated in an incubator/shaker (New Brunswick) at 37°C >200 rpm). During incubation, the growth was followed by measuring the optical density of the culture at 600 nm (OD600). At OD600=0.5, the culture was transferred to an ice/water bath for 10 min before induction of protein expression by adding isopropylthiogalactoside (IPTG) to 0.5mM final concentration. An un-induced sample was grown in parallel and both cultures were incubated in an incubator/shaker set at 25°C for 6 hours before harvest. Cells were harvested by centrifugation, and the pellet was resuspended in 30 ml lysis buffer [50 mM Tris-Cl pH=8.0 (4 °C), 300 mM NaCl, 5% Glycerol, 1mM DTT, 1x Protease inhibitor cocktail] and lysed by French press as

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described above. The lysate was cleared by centrifugation (15k rpm, 30 min, 4 °C, SS34 rotor) and loaded onto 1 ml GST-sepharose equilibrated in lysis buffer. Resin was washed and GST fusion protein was eluted in fractions of 1 ml each, using lysis buffer containing 10 mM reduced glutathione (adjusted to a pH = 8.5 with NaOH). A gel of this step in the purification is shown in Figure 7. Induction was efficient (compare lane 1 & 2) and the majority of the Ess1-GST protein was soluble (compare lane 2 & 3). Some overloading of the column is seen by the amount of GST-Ess1 flowing through the column (lane 5). Elution was efficient as evidenced by the prominent Ess1-GST band in the elution fractions (lane 9 – 13).

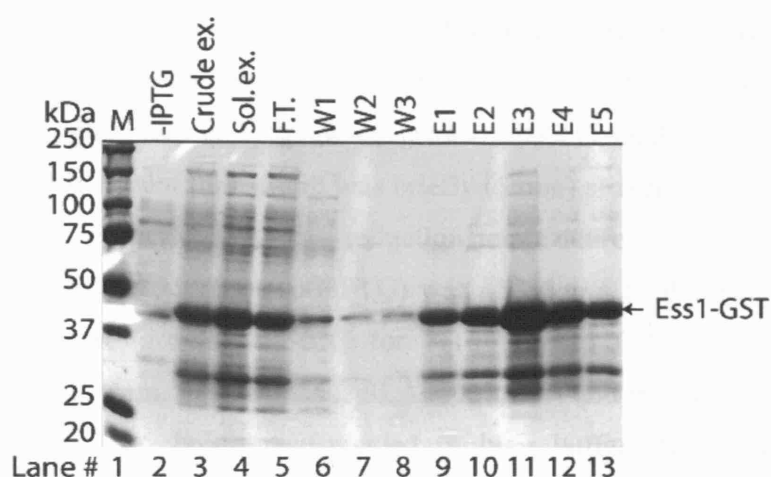


Figure 7 Analysis of Ess1-GST purification.

*Coomassie stained 10% SDS-PAGE. Ess1 was purified by Glutathione-S-transferase affinity chromatography. Samples from each of the individual steps of the procedure were analysed. Abbreviations: -IPTG: uninduced, + IPTG: induced to 0.5 mM IPTG, F.T.: unbound, W: wash fraction, E: Elution fraction*

After the GST column, several contaminating bands remained in the preparation (lane 9 -13). Consequently, it was decided to purify the protein further by Mono Q chromatography. The pooled protein was diluted in buffer A0 [50 mM TrisCl pH 7.8 (4°C), 1mM EDTA, 10mM DTT, 20% glycerol, Protease inhibitors] until the conductivity was less than 25mM KCl. The MonoQ HR 5/5 column (GE Healthcare) was loaded and run at 0.3 ml/min at 4°C. The column was washed and developed with a salt gradient from 25 mM to 1.4 M KOAc while collecting 0.5 ml fractions. The gel is shown in the results section.

### 2.9.5 Purification of GST-CTD

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In order to test whether the purified Mediator would interact with a GST-CTD protein and to investigate the effect of CTD phosphorylation on the interaction, it became necessary to purify GST-CTD. A plasmid (pGEX-4-T1, Amersham Biosciences) expressing glutathione-S-transferase fused to the yeast CTD sequence encompassing 26 repeats was obtained from the Proudfoot lab (described in (Barilla et al., 2001)). The fusion protein was transformed by electroporation in a 2 mm cuvette (Equibio), containing 50  $\mu$ l competent cells at 1.5 kV, 25  $\mu$ F, 600 ohm resistance, time constant  $\sim$ 12, Bio-Rad apparatus) in BL21(DE3) cells. Transformants were selected on LB agar plates, containing 100  $\mu$ g/ml Ampicilin, streaked to single colonies and used to inoculate an overnight culture of 1/100 the volume of the final culture. For overnight cultures, Luria Broth (LB) medium containing 100  $\mu$ g/ml Ampicilin was normally used (at 37°C). Pre-warmed medium was inoculated the following day with at least 1/100 volume of overnight culture. Cells were grown at 37°C until just before reaching  $OD_{600}$  = 0.6, at which point the culture was briefly (5 min) shaken in a ice-water bath to rapidly lower the temperature to the induction temperature of 30°C. At this point, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and cells were grown at 30°C for 3-16 hours. Cells were harvested by centrifugation (9000 rpm, 4°C, 10 min, SLA3000 rotor) and the pellet was washed in ice cold H<sub>2</sub>O before being resuspended in lysis buffer. The GST step of the purification was analysed by 10% SDS-PAGE and the analysis is shown in Figure 8. After the GST step, some contaminating bands still persisted. These formed an evenly-spaced ladder of bands below the full length band (Figure 8 lanes 10 & 11) possibly reflecting premature translational termination possibly due to lack of specific cognate tRNA species. A similar smear has previously been observed during GST-CTD purification (Peterson et al., 1992). In addition, three large bands presumably arising from GST-CTD protein degradation products were visible at 25 kDa (Figure 8, lanes 10 & 11). GST-CTD should theoretically migrate as a 47 kDa protein but its tendency to migrate as a 65 kDa protein has been reported by others (Morris et al., 1997).

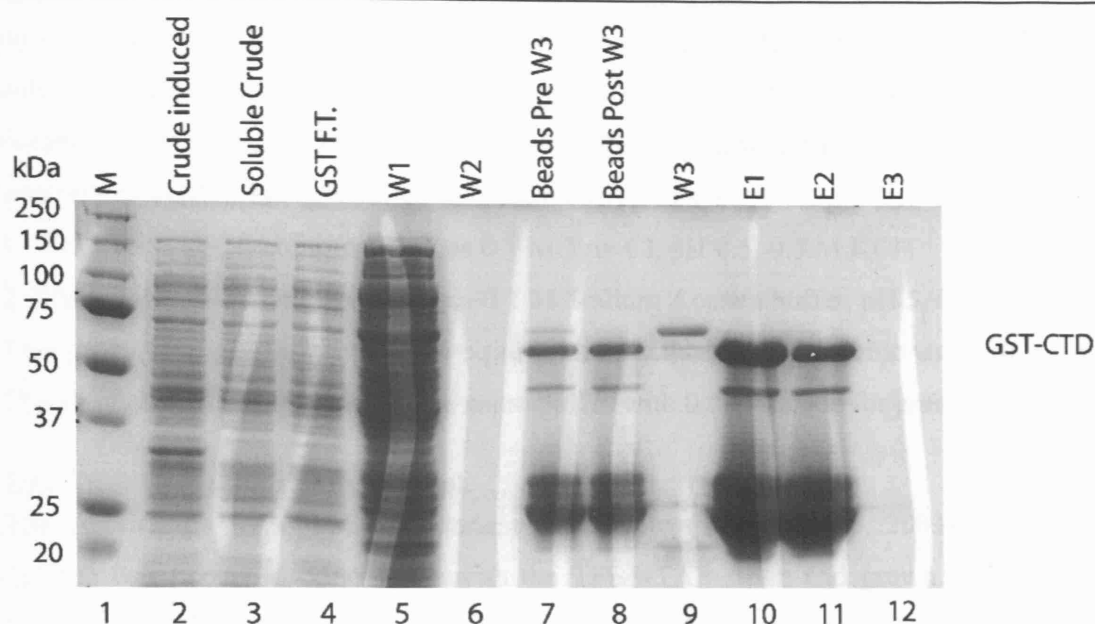


Figure 8 GST-CTD purification GST step

10 % SDS-PAGE analysis of GST purification. Abbreviations are: W: Wash, E: Elution. Protein from 1 ml of Wash1 was precipitated using Strataclean resin and loaded (lane 5). In lane 6 -12 10  $\mu$ l of the designated samples were loaded pr. lane.

The band at ~70 kDa (lane 7) is probably the *E. coli* chaperone DnaK, which is a common contaminant in GST fusion protein purification. According to the instructions of the resin manufacturer (GE Biosciences), DnaK can be dislodged from the protein of interest by washing in a buffer containing [50 mM Tris-HCl, 2 mM ATP, 10 mM MgSO<sub>4</sub>, pH 7.4 for 10 min. at 37°C] (Wash 3 buffer). Beads were loaded before (lane 7) and after batch incubation with 1.5 column volume Wash 3 buffer. Strataclean extraction of the wash (lane 9) shows predominant elution of DnaK. To purify the GST-CTD protein further, I pooled the elution fractions and dialysed them against buffer B<sub>50</sub>, and applied them to a MonoQ HR 5/5 column. This procedure is described above.

#### 2.9.6 Heparin affinity purification

Heparin columns were packed from re-generated Heparin-sepharose aiming to use 1 ml Heparin bed volume for each 60 mg of crude yeast protein loaded onto the column. The flow-rate was maximally 2 column volume/hours and the column was normally mounted in a Bio-Rad Biologic system. Before the run, the heparin column was equilibrated in buffer K-buffer: [50 mM Tris-Cl, pH 7.9; 20% glycerol; 1 mM EDTA; 1 mM DTT; 1 x protease inhibitors. KCl was added to the mM concentration indicated by the subscript]. Crude protein extract was loaded directly onto Heparin-Sepharose and

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protein was eluted from the column by washing with 2 column volume buffer K<sub>200</sub>, 3 column volumes buffer K<sub>600</sub>, and 3 column volume buffer K<sub>2000</sub>. During the run, 1/10 column volume fractions were collected and the A<sub>280nm</sub> was monitored. Used heparin was regenerated in the column by two consecutive washes:

1. Wash with 15-20 column volumes 0.1 M Tris-Cl, pH 8.5, 0.5 M KCl
2. Wash with 15-20 column volumes 0.1 M Sodium Acetate buffer, pH 5, 0.5 M KCl.

This procedure was followed by re-equilibration with at least 5 column volumes K-100. The Heparin resin was stored in the same buffer with 0.02 % azide for preservation.

### 2.9.7 Tfb3-TAP purification protocol.

The purification of TFI<sub>IK</sub> was done according to (Takagi et al., 2003). 12 litre of 2xYPD was prepared, inoculated with the TFB3-TAP strain and grown at 30°C with shaking until stationary phase (at  $6.2 \times 10^8$  cells/ml), whereupon cells were harvested by centrifugation at 6000 rpm (4°C, 10 min, Beckmann, SLC-6000 rotor) and resuspended in ice cold H<sub>2</sub>O. Cells were extruded into liquid nitrogen and lysed under liquid nitrogen in the freezer mill (5 cycles of 2 min milling/2 min cooling, at 13 impacts pr second), and the resulting yeast powder was stored at -80°C until use.

On the day of use, the pellet was thawed in 1.45 ml lysis buffer (see formulation below) pr. gram cells by incubating 30 min on a roller (4°C). All steps from this point on were done at 4°C. The lysate was pre-cleared from crude debris by centrifugation (SLA3000, 14k rpm, 20 min at 4°C) and subsequently re-cleared by ultracentrifugation (Ti 45, 42k rpm, 90min, at 4°C). Proteins were precipitated by adding powdered (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> slowly over the course of 30 min to 60% saturation. The lysate was left to equilibrate with gentle stirring for a further 60 min and the pellet was collected by centrifugation (SLA1500, 10,5K rpm 50 min, 4°C). The pellet was dissolved in H<sub>0</sub> buffer and the lysate was further diluted until conductivity was below that of 0.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at which point the lysate was cleared by ultracentrifugation (Beckman Ti45, 40k rpm, 4°C, 1 hour, deceleration profile 9) and divided into aliquots of 50 ml per tube.

The IgG resin was prepared by two cycles of washing (10 column volumes. TST and 10 column volumes 0.5 M HOAc). The resin was washed in H<sub>500</sub> buffer and re-pelleted until the supernatant pH = 7.6. For each 50 ml tube of extract, 0.5 ml bed volume of IgG resin was added and the mixture was incubated on a roller (2 hours at 4°C). The resin was collected by gentle spinning (Beckman GH 3.8 rotor, 500 rpm, 4°C, 2 min) and the supernatant was filtered through a 10 ml poly prep column (Bio-Rad cat. no 731-1550),



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or a 20 ml Econo-Pac column (Bio-Rad cat. no. 732-1010). The beads were transferred by gentle re-suspension and pipetting and the tip and 50 ml tube was washed with the flow through from the poly-prep column to collect all resin. Lastly, a frit was mounted (Econo-pac columns) and placed gently on the resin bed. The column was washed with 25 column volume  $H_{500}$  + protease inhibitors, then 25 column volumes  $H_{100}$  without protease inhibitors, and lastly with 25 column volumes ml Tev-protease buffer.

The tagged protein was eluted by addition of 2 column volumes protease buffer with Tev protease (ca. 100 units per column volume). One column volume flow-through was immediately collected and the column was closed (top and bottom). For enzymatic digestion, the column was left standing over night at 5 °C. For elution, 5 column volume protease buffer was added and eluate was collected in 1 column volume fractions.

### **Buffers:**

#### **1x Lysis buffer:**

270 mM	Tris Acetate pH=7.6 @ 4°C
1 M	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>
90 mM	KOAc
1.8 mM	EDTA
18%	glycerol.
10 mM	β-mercaptoethanol

Protease inhibitors.

#### **H<sub>0</sub> buffer**

25 mM	Hepes pH 7.6 @ 4°C
0,1 mM	EDTA
15 %	glycerol
5 mM	β-mercaptoethanol

Protease inhibitors.

#### **Tev protease buffer:**

50 mM	Hepes-KOH pH= 8.0 @ 4°C
0.1 mM	EDTA
200 mM	KOAc
5 mM	β-mercaptoethanol
10%	glycerol

### **2.9.8 Purification of CTDK-1 kinase**

The procedure for purification of CTDK-1 kinase was based on (Sternier et al., 1995). Open Biosystems CTK1-TAP strain (YSC1178-7501481) was grown in 12 litres 2xYPD to a density of  $1 \times 10^8$  cells/ml and harvested as described above. Whole cell extract was made by freezer milling (5 cycles disruption/pre-cooling of 2 min each,

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impacts set at 13 hits pr second) of yeast cells harvested from 12 litre of late-log phase culture in YPD. The powdered extract was thawed at 4°C and cleared by centrifugation (Sorvall SLA1500, 14k rpm, 30 min, 4°C).

To precipitate DNA fragments and associated proteins, 10% PEI (pH 7,9) was added drop-wise to a concentration of 0.0175% . With a magnet, the solution was gently mixed for 30 minutes on ice. The precipitate was pelleted (Sorvall SLA1500 rotor, 10k rpm, 15 min @ 4°C) and the supernatant discarded. TAP buffer (35 ml) was added and the pellet was scraped into a pre-cooled 40 ml Dounce homogeniser. The pellet was homogenized in ice by >20 strokes using the “A” pestle. The lysate was pelleted again (Sorvall SLA1500, 14k rpm, 30 min, 4°C) and the supernatant discarded. For elution of protein from DNA/chromatin, 40 ml ice-cold TAP<sub>400</sub> buffer was added and the pellet was homogenised in a clean, cold, Dounce homogenizer as before. The pellet was collected (Sorvall SLA1500, 12k rpm, 30min, 4°C) and the supernatant saved. To precipitate protein, freshly ground solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was slowly added to 40% saturation, and the solution was gently mixed with a magnet (30-60 min, 4°C). The precipitate was collected by centrifugation (Sorvall SS34, 14k rpm, 40 min, 4°C). At this point, the supernatant was removed, and the procedure was either continued or the pellet was stored on ice overnight.

H<sub>0</sub> buffer was added to resuspend the pellet until conductivity was below that of 0.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the solution was cleared by centrifugation (Beckman Ti45, 40k rpm, 4°C, 1 hour, deceleration profile 9). The supernatant was aliquoted into 50 ml tubes and 0.5 ml bed volume of IgG resin was added per 50 ml tube. Prior to protein loading, the resin was prepared in TST/0.5 M HOAc as per manufacturer protocol and then equilibrated in H<sub>500</sub>.

Beads and proteins were incubated on a roller for 2 hours at 4°C and the resin was collected by gentle spinning (Beckman GH 3.8 rotor, 500 rpm, 4 °C, 2 min). The supernatant was filtered through a 10 ml Bio-Rad poly-prep column, and then collected by gentle spinning. Tubes were washed with flow-through to collect all beads. Resin was initially washed with 25 column volume H<sub>500</sub>+ protease inhibitors, and then with 25 column volumes H<sub>100</sub> without protease inhibitors. Finally, the resin was washed with 25 column volumes TEV-protease buffer without protease inhibitors. Protein was eluted by adding 2 column volumes of TEV-protease buffer containing 100 units TEV protease per column volume. One column volume flow-through was collected before closing the column (top and bottom). The column was left at 4°C overnight. Next day, 5 column

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volumes of protease buffer was added and eluate collected in 1 column volume fractions.

### **Buffers:**

#### **2 x Lysis buffer.**

200 mM Hepes pH=7.8 @ 4°C  
100 mM KOAc  
20 % glycerol  
2 mM EDTA  
10 mM DTT  
2 x Protease inhibitors.

#### **TAP buffer.**

20 mM Hepes pH=7.8 @ 4°C  
50 mM KOAc  
10 % glycerol.  
1 mM EDTA  
10 mM DTT.  
Protease inhibitors,

#### **H<sub>0</sub> buffer**

25 mM Hepes pH 7.6 (4°C)  
0,1 mM EDTA  
15 % glycerol  
5 mM β-mercaptoethanol  
Protease inhibitors.

#### **Tev protease buffer:**

50 mM Hepes pH= 8.0 (4°C)  
0.1 mM EDTA  
200 mM KOAc  
5 mM β-mercaptoethanol  
10 % glycerol.

KCl was added to the concentration indicated by the subscript.

## ***2.10 Ion exchange chromatography***

### **2.10.1 Bio-Rex 70 chromatography**

Bio-Rex70 columns were run in buffer A [20 % glycerol, 40 mM Hepes pH 7.6, 1 mM DTT, 1 mM EDTA, 1 x protease inhibitors, with potassium acetate (KOAc) as indicated by subscript]. The columns were packed in Bio-Rad column holders (I.D. 5 cm) from regenerated Bio-Rex70, using 1 ml of resin for each 60 mg of crude yeast protein to be loaded. Resin was degassed before use and the column was mounted in a Biologic

FPLC system (Bio-Rad) and packed under low flow of  $A_0$ . The re-used resin was washed with 2 column volumes  $A_{1200}$ , before equilibrating in  $A_{150}$ . Columns were run at 2 column volume/hour. Crude yeast extract was cleared from debris by centrifugation (Sorvall SLC-6000 rotor, 8000 rpm, 4 °C, 30 min) and filtered through 4 layers of gauze, before application to the column. Unbound protein was removed with 1 column volume  $A_{150}$ . The column was developed by step elution using 2 column volumes  $A_{300}$ , 2 column volumes  $A_{600}$ , and 2 column volume  $A_{1200}$ . The  $A_{280nm}$  absorbance of the effluent was monitored and 1/10 column volume fractions were collected. After the run, the column was regenerated with 3 column volumes 0.5 M KOH. The column was then rinsed with 4 column volumes MilliQ water, and saved in 40 mM Hepes pH 7.6, 1 mM EDTA, 0.02 % azide.

### 2.10.2 DEAE Sephacel chromatography

DEAE Sephacel columns were run in buffer B [20 % glycerol, 20 mM Tris-Acetate pH 7.8, 1 mM DTT, 1 mM EDTA, 1 x protease inhibitors, Potassium Acetate (KOAc) as indicated by the subscript]. In the holoRNAPII purification procedure, a binding capacity of 20 mg protein per ml of DEAE Sephacel resin was assumed. The column was packed under pressure from regenerated DEAE Sephacel and washed in 2 column volume  $B_{1000}$  before equilibration in  $B_{100}$ . Speed of run: 2 column volumes per hour, collecting 1/10 column volume fractions. The protein was loaded and unbound protein was removed by a 1 column volume wash. The column was developed with step elution using 2 column volumes  $B_{200}$ , 2 column volumes  $B_{550}$  (designated  $DE_{550}$ ) and two column volumes elution at  $B_{1000}$ . After the run, the column was flushed with  $H_2O$  and washed with 3 column volume 0.5 M KOH. It was then rinsed with 4 column volumes MilliQ water until pH was near neutrality, and saved in 20 mM Tris-Acetate pH 7.8, 1 mM EDTA, 0.02 % azide.

### 2.10.3 Hydroxylapatite chromatography

Hydroxyapatite (HAP) columns were packed based on the amount of protein to be loaded. The hydroxyapatite binding capacity for yeast protein is 5-8 mg protein per ml resin (Bio-Gel HTP Hydroxyapatite, Bio-Rad cat. # 130-0421). To prepare buffers, a 1 M stock phosphate buffer was made by adjusting the pH of 1M  $K_2HPO_4$  with 1M  $KH_2PO_4$  at 4°C with stirring until pH=7.7. The phosphate stock and  $H_2O$  was added to 2x concentrated  $C_0$  (1x is [20 % glycerol, 100 mM KOAc, pH 7.5-7.8, 1 mM DTT, 0.1 mM EDTA, 0.01 % NP-40]) to make  $C_{10}$  and  $C_{200}$ , and protease inhibitors were added

to 1x just before use. An appropriate amount of HTP hydroxyapatite (1 g gives 2-4 ml resin) was dissolved in 5-10x the volume (1 ml/g HAP) of cold, C<sub>10</sub> buffer. To remove fines, resin was degassed on ice until settled (5-10 min) and the supernatant was poured off. This was repeated a total of 3 times. Finally, the hydrated HAP resin was re-dissolved in C<sub>10</sub> buffer and the desired amount was poured into a Bio-Rad column cartridge. Equilibration, load, and wash were done at 2 column volume /hour, but the gradient elution was run at 1 column volume/ hour only. The column was equilibrated with one column volume C<sub>10</sub> buffer before protein was loaded. After loading, the column was washed with 1 column volume C<sub>10</sub>. The column was developed with a 10 column volume gradient from 10 mM to 200 mM phosphate in buffer C while collecting fractions of 1/4 column volume pr fraction.

### 2.10.4 MonoQ Chromatography

Preparative MonoQ chromatography was done using pre-packed columns from GE biosciences. Columns of 1 ml bed volume were: Mono Q HR 5/5. (cat. no. 17-5167-01) and MonoQ 5/50 GL Tricorn (cat no. 17-5166-01). These were mounted in an AKTA FPLC system. For analytical purposes, a Smart HPLC system was used with a MiniQ PC 3.2/3) column (cat. no. 17-0686-01) of 100 µl bed volume.

Buffers depended on the protein to be purified, but for MonoQ resin typically consisted of a Tris-Cl buffer (20 -50 mM) buffered at the operating temperature and degassed. All buffers were filtered through 0.2 µm pore size filters before use. Columns were equilibrated in 10 column volume of buffer before loading of protein. All protein samples to be loaded were cleared by high speed centrifugation (20min, 20000 rpm, SS34 rotor, 4 °C) immediately before loading. The flow rate was dictated by the backpressure generated and the pressure limits of the column. The column was washed in low salt buffer until the light absorption at 280 nm ( $A_{280}$ ) of the column effluent was at baseline. From this point, it was developed in 10 column volume from 100 mM KCl to 1.2 M KCl collecting 1 – 0,5 column volume fractions. Finally, a 2M KCl cleaning step was performed for 5 column volume before the column was re-equilibrated in low salt buffer. After use, the backpressure of the column was checked and, if needed, cleaning measures were undertaken before storing the column in 20% ETOH.

### **2.10.5 Phosphocellulose (P11) chromatography**

P11 resin (Whatman cat. no. 4071-050) was always prepared no more than 24 hours before single-use. To hydrate the resin, 0.33 grams of phosphocellulose powder per ml required resin was weighed. The powder was stirred into 400ml 0.5 M KOH and immediately pelleted in a 0.5 litre and centrifuge tube (Sorval SLA1500 rotor, 2000 rpm, 1 min, 4 °C). The supernatant was decanted and resuspended in MiliQ H<sub>2</sub>O, and pelleted as above until indicator paper showed the pH of the supernatant to be below 11. At this point, the pellet was resuspended in 400 ml 0.5 M HCl and pelleted (Sorval SLA1500 rotor, 3000 rpm, 1 min, 4 °C). Resin was washed repeatedly in H<sub>2</sub>O as above until the pH was above 3. Finally, the resin pellet was resuspended in 5 column volume A<sub>600</sub> containing 200 mM Hepes pH 7.6, and the pH of the slurry was finely adjusted to pH 7.6 with 0.5 M KOH before pelleting and resuspension in A<sub>0</sub> buffer. The resin was degassed and poured into a Bio-Rad column holder where it was packed under pressure and equilibrated in 150 mM KOAc.

For the TFIIF purification procedure, the binding capacity of phosphocellulose was taken as 20 mg protein/ml resin. The column was mounted on a Bio-Rad Biologic system at a flow of 2 column volume/hours. For a standard run the program was: Load protein in 150 mM KOAc, then 1 column volume wash with A<sub>150</sub> and two elution steps using 2 column volume A<sub>300</sub>, followed by 2 column volume elution with A<sub>600</sub>. Fraction of 1/10 column volume were collected and fractions were analysed by Bradford and pooled if the protein concentration was more than 2 mg/ml. The protein was then loaded directly onto Ni-NTA affinity resin for further purification.

### **2.10.6 Gel filtration chromatography**

Gel filtration columns were stored at 4°C in 20% ethanol. All solutions were filtered through a 0.22 µm pore size filter (Nalgene) and degassed at 4°C for 10-20 min under vacuum (with stirring), just before use. To start a run, the EtOH was flushed from the column with H<sub>2</sub>O for 2 column volume at a minimal flow-rate to avoid high backpressure. The column was equilibrated in >5 column volume of running buffer before loading protein. Calibration of columns was performed before and after each sequential batch of runs, first using blue dextran (MW~ 2000 kDa) for void volume determination and a gel filtration standard kit ( Bio-Rad cat. # 151-1901), containing a mix of proteins (see Table 1) for determination of the lower range of MW separation.

Table 1 MW standards for gel filtration calibration

Protein	MW	pI
Thyroglobulin	670,000	4.5
Bovine gamma-globulin	158,000	5.1
Chicken ovalbumin	44,000	4.6
Equine myoglobin	17,000	6.9
Vitamin B12	1,350	4.5

Size exclusion chromatography was carried out in salt concentrations equal to or above 250mM KOAc to avoid non-specific protein-protein and protein-resin interactions. Running buffer composition depended on the protein to be analysed, but as a general rule, buffers never contained glycerol, but always a low concentration (0.01% - 0.05%) of detergent (Nonylphenyl-polyethylene glycol (NP-40), Polyoxyethylene sorbitan monolaurate (Tween-20) to reduce non-specific hydrophobic interactions. The load volume was maximally 3% of the column volume and between runs, the column was always washed with at least 2 column volume of running buffer. The flow-rate used depended on the column and the back-pressure generated under the specific buffer conditions. The Superose 6 PC 3.2/30(Amersham Biosciences) column for the SMART system (Amersham Biosciences) has a column volume of 2.4 ml and a theoretical separation range from 5 kDa - 5000 kDa (manufacturer instructions) and was maximally loaded with 50µl of sample. Runs were done at 40µl/min, 5°C, fractions of 50µl were collected.

The TSK G4000SW (Tosoh Bioscience) gel filtration column has a column volume of 26.5ml and a theoretical separation range of 20 kDa to 7000 kDa (manufacturer instructions) and was loaded with maximally 0.5 ml sample. The column was mounted on either an AKTA FPLC (Amersham Biosciences) or a Biologic FPLC (Bio-Rad) system, equilibrated in running buffer for 5 column volumes and run at 0.3 - 0.5 ml/min at 4°C while collecting fractions equal to the load volume in size. The column was washed in 3 column volume filtered H<sub>2</sub>O, before being stored in 2 column volumes 20% EtOH at 4°C.

### 2.10.7 Purification of coreRNAPII

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For coreRNAPII purification, a culture of the protease deficient yeast strain BJ2168 was grown to late-log phase in a batch fermentation vessel. After harvesting, the cell pellet was resuspended in half its volume of 3X lysis buffer K100) [1X K100-buffer: 50 mM Tris-Cl, pH 7.9; 100 mM KOAc; 20% glycerol; 1 mM EDTA; 1 mM DTT; 1 x protease inhibitors (the subscript after K denotes the KOAc concentration)] and lysate was made as described above and frozen in liquid nitrogen. The lysate was thawed in lukewarm water with stirring until 80% of each tube was liquid, then put directly on ice and pooled. All handling from this step on was done in the cold room. Protein content was measured by a Bradford based method using a Bio-Rad kit and a corresponding volume of regenerated heparin-sepharose was packed into a Bio-Rad column holder (5 cm diameter) using 1 ml of heparin-sepharose per 60 mg of crude soluble protein. The packed column was connected to a Bio-Rad Econo column pump system and equilibrated in 4 column volume of  $K_{100}$  before loading protein at 2 column volume/hour and washing unbound protein away with  $K_{100}$  to near baseline UV absorption (at 280nm). Subsequently, the column was washed in 2 column volumes  $K_{200}$ , eluted in 2 column volume  $K_{600}$  and washed in 2 column volume  $K_{2000}$  before being regenerated. During  $K_{600}$  elution, fractions of 1/10 column volume were collected. The peak fractions from the  $K_{600}$  elution were pooled and protein was precipitated by addition of powdered solid  $(\text{NH}_4)_2\text{SO}_4$  to 50% saturation. The pellet was collected by centrifugation at 40k rpm (Ti45 rotor, Beckman) at 4°C for 1 hour and gently resuspended in TEZ<sub>0</sub> buffer [1x TEZ<sub>0</sub> :10 mM Tris-Cl pH=7.6; 1 mM EDTA; 0.01 mM  $\text{ZnCl}_2$  ; Protease inhibitors] (number in subscript denotes the  $(\text{NH}_4)_2\text{SO}_4$  concentration in mM)] until a conductivity corresponding to 500 mM  $(\text{NH}_4)_2\text{SO}_4$  was reached. The protein was applied to 5 ml of protein A-sepharose conjugated to 8WG16 antibody in a 20 ml Bio-Rad Econo column holder and incubated at 4°C overnight on a roller table. The 8WG16 antibody recognizes the hypophosphorylated CTD repeats of Rpb1 and its binding is sensitive to high salt concentration in combination with polyols, which enables the elution of specifically bound proteins with 50% to 70% of glycerol or ethylene glycol and 0.5 M  $(\text{NH}_4)_2\text{SO}_4$  (Thompson and Burgess, 1996). After loading, the 8WG16 resin was washed with 100 column volume of TEZ<sub>500</sub> and protein was eluted from the column using a slow flow (1/10 column volume/min) of TEZ<sub>500</sub> containing 50% glycerol (Thompson et al., 1990). Eluted protein was diluted with B<sub>0</sub> [40mM Tris-CL pH=7,6, 1 mM EDTA; protease inhibitors (subscript number denotes the concentration of KOAc in mM)] to a conductivity less than 100mM KOAc and



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cleared by ultracentrifugation at 40k rpm (Beckman Ti45 rotor, 4°C, 60 min). A DEAE-5PW HPLC column (3.3 ml column volume, Tosho Bioscience cat. 07164) was connected to a AKTA FPLC system (Amersham) and equilibrated in B<sub>100</sub>. The entire run was done at 0.3 ml/min and after loading of the protein, the column was washed to baseline A<sub>280nm</sub> absorption with B<sub>100</sub> before developing with a 13 column volume gradient from 0.1 to 1 M KOAc in B buffer while collecting fractions of 0.3 ml. Fractions were analysed by 10% SDS-PAGE and Coomassie staining to identify peak RNAPII fractions. Peak fractions were pooled and dialysed against Buffer A<sub>100</sub> for 3 – 4 hours at 4 °C. Finally, RNAPII was frozen in liquid nitrogen and stored at -80 °C. Typically, concentrations were from 100 – 300 ng/μl.

### 2.10.8 Purification of holoRNAPII

Yeast lysate from 50 liters of culture was thawed as described above and the total protein concentration was measured by a Bradford-reaction based kit (Bio-Rad cat.no. 500-0006EDU). Regenerated Biorex-70 was packed into a large column holder (Bio-Rad, 5 cm inner diameter or ID), using 1 ml Biorex-70 per 100 mg whole cell protein to be loaded. The column was gently packed under flow and equilibrated in 3 column volumes A<sub>100</sub> buffer. The whole cell extract was applied to the Biorex70 and unbound protein was removed by washing with 1-2 column volumes A<sub>100</sub> buffer. Subsequently, the column was step eluted with 2 column volume A<sub>300</sub>, 2 column volumes A<sub>600</sub> and 2 column volumes A<sub>1200</sub>. Fractions of 1/10 column volume were taken when running smaller (<100 ml) Bio-Rex70 columns. For larger columns, the A<sub>280nm</sub> value was monitored for peaks and fractions were collected accordingly. The peak of HoloRNAPII eluted with A<sub>600</sub> buffer. This peak of the B<sub>600</sub> elution (BR<sub>600</sub>) was dialysed against buffer B<sub>25</sub> [20 % glycerol 20 mM Tris-Acetate pH 7.8, 1 mM DTT, 1 mM EDTA, Potassium Acetate (KOAc) as indicated by subscript] until the conductivity was less than B<sub>100</sub>, at which point the preparation was cleared at 40k rpm (Beckman Ti45, 4°C, 1 hour) and the concentration of soluble protein determined, using a Bradford based assay from Bio-Rad.

The DEAE column was packed in a Bio-Rad column holder (2 cm inner diameter), using 1 ml regenerated DEAE-Sephacel per 20mg of protein in the BioRex<sub>600</sub> elution and the column was equilibrated in B<sub>100</sub> (approximately 5 column volumes). The cleared BioRex<sub>600</sub> elution was dialysed or diluted until the conductivity was below that

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of B<sub>100</sub>, and loaded onto DEAE-Sepharcel. Unbound protein was removed by washing in 1-2 column volumes B<sub>100</sub>. Next, the column was eluted using 2 column volumes B<sub>200</sub>, 2 column volumes B<sub>550</sub> and 2 column volume B<sub>1000</sub>. Fractions of 1/10 column volume were collected throughout. HoloRNAPII normally peaks in the B<sub>550</sub> elution (DE550). The regeneration of DEAE followed the regeneration of BioRex70 (see above) apart from storing the regenerated column in B<sub>0</sub> instead of A<sub>0</sub>. The peak of holoRNAPII subunits was identified through immunoblotting of the elution profile (see figure 15), pooled, and loaded on a hydroxyapatite column.

The HAP was prepared as described above under “Ion exchange chromatography”. Pooled holoRNAPII-containing fractions from the DEAE STEP were loaded directly onto HAP equilibrated in buffer C<sub>100</sub>. The column essentially bound all the applied protein and after 1 column volume wash in C<sub>100</sub> buffer the column was developed with a linear gradient of 10-200 mM inorganic phosphate over 10 column volumes at 0.1 ml/min and collecting fractions of 1/4 column volume. Samples from the elution profile were analysed by 10% SDS-PAGE and immunoblotted to identify the fractions containing RNAPII and Mediator subunits, as well as coreRNAPII, Elongator, and TFIIF subunits. RNAPII holoenzyme peak fractions were pooled, aliquoted, and frozen in liquid N<sub>2</sub>. Protein was stored at -80°C

### **2.10.9 Gel filtration analysis of HAP fraction # 24 containing holopolymerase**

A Superose 6 column (Amersham) was installed in an FPLC system (SMART, Amersham) and equilibrated in 1x gel filtration buffer [40 mM Hepes, 15 mM Imidazole, 0.02% Tween-20, 0.01% NP40, 5mM β-Me, and either 250 mM KOAc or 400 mM KOAc]. Fractions of 50 µl were collected. The UV<sub>280nm</sub> absorption profile of gel filtration markers (Bio-Rad cat. no 151-1901) demonstrated the distribution of apparent molecular weights in the various fractions: (thyroglobulin (670kDa – fraction number 28), bovine gamma-globulin (158 kDa fraction number 34), chicken ovalbumin (44kDa –fraction 36-37), equine myoglobin (17kDa – fraction number 39) and vitamin B12 (1.35 kDa - fraction 42-43)).

This analysis also revealed that a peak corresponding to aggregates of protein markers (according to manufacturers instruction) emerged from the column in fraction 9 indicating the end of the void volume and the beginning of the separation range, which for Superose 6 is from 6000 kDa to 1.3 kDa (manufacturer instruction). The column was loaded with 50 µl HAP #24 fraction and run at 30 µl/min at 4°C.

### 2.10.10 Purification of holoTFIIH

The procedure consists of two steps of ion exchange chromatography (Biorex-70 and phosphocellulose chromatography), followed by an affinity chromatography step to isolate Tfb1-6His tagged holoTFIIH and a MonoQ step to concentrate and further purify it as described in (Svejstrup et al., 1994). All steps were conducted on ice or at a maximum temperature of 4 °C. Buffers were degassed before use and the pH was adjusted to 4 °C. Yeast extract (corresponding to 50 litre culture) was thawed in lukewarm water and KOAc was added to 0.5 M to extract proteins from chromatin with gentle stirring. After 30 min incubation at 4 °C, slow addition of 10% PEI (adjusted to pH=7.9) to a final concentration 0.1% served to precipitate polymer nucleic acids. After gentle stirring for a further 30 min the extract was cleared by ultracentrifugation (45Ti rotor (Beckmann), 42000 rpm, 90 min, 4 °C) and the supernatant was diluted in A<sub>0</sub> buffer (20% glycerol, 40 mM Hepes pH=7.6, 1 mM EDTA, 1mM DTT, 1x protease inhibitors, KOAc to indicated subscript in mM) to a conductivity corresponding to A<sub>200</sub>. A Biorex70 column was packed into a BioRad column holder (5 cm ID) using 1 ml bed volume for each 60 mg protein in the crude extract and equilibrated in A<sub>200</sub>. The diluted crude extract was loaded at 2 column volume/ hour and the column was washed in 1 column volume A<sub>200</sub> and 2 column volumes A<sub>300</sub> and finally the column was step-eluted using 2 column volumes A<sub>650</sub>, while collecting 1/10 column volume fractions. The peak of protein as judged from the absorption at 280 nm was pooled and dialyzed against A<sub>0</sub> until the conductivity was that of A<sub>200</sub>. Next, the protein was cleared by centrifugation (Beckmann Ti45 rotor, 42000rpm, 30 min 4 °C) and applied to a phosphocellulose (Whatman) column (packed using 1 ml bed volume for each 20 mg protein eluted from BioRex70) pre-equilibrated in I<sub>200</sub> (20% glycerol, 40mM Hepes pH=7.6, 10 mM Imidazole, 0.2% Tween-20, 0.01% NP40, 5mM β-mercaptoethanol, 1x Protease inhibitors, and KOAc to the concentration (in mM) indicated by the subscript after I). The column was loaded and run at 2 column volume/hour in a Biologic FPLC system (Bio-Rad) and developed using a 5 column volume gradient from 200 mM KOAc to 800 mM KOAc while collecting 1/10 column volume fractions. The peak was detected by a Bio-Rad Bradford assay of fractions and pooled. A column holder (0.7 ml ID, Amersham) was packed with 1 ml of NiNTA Superflow FF (Qiagen), mounted in an AKTA FPLC system, and equilibrated in I<sub>500</sub>. The pooled protein from phosphocellulose was loaded at 0.1 ml/min by recirculation overnight at 4°C. Next, the column was washed at 0.02 ml/min, first with 10 ml buffer J<sub>1000</sub> (20 mM Tris-acetate,

## Materials & Methods: Protein Purification Procedures

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pH 7.6, 20% glycerol, 5mM  $\beta$ -mercaptoethanol, 1x protease inhibitors, KOAc (in mM) as indicated by subscript after J) and then with 10 ml buffer J<sub>400</sub>, containing 20 mM Imidazole. The column was eluted in batch using J<sub>400</sub> containing 250 mM Imidazole and the elution fractions were tested for CTD kinase activity. Active fractions were pooled and diluted with B<sub>0</sub> (20 mM Tris-Acetate pH=7.8, 1mM DTT, 1mM EDTA, 1x Protease inhibitors, KOAc as indicated by the subscript after B) to a conductivity corresponding to B<sub>200</sub> and loaded onto a MonoQ HR 5/5 column (Amersham) equilibrated in B<sub>200</sub>. Unbound protein was removed with 2 column volume B<sub>200</sub> and the column was developed in 10 column volume from 200 mM to 1M KOAc at 0.3 ml/min while collecting fractions of 250  $\mu$ l each. The peak fraction was identified by immunoblotting using antibodies against Ccl1 and the 6xHis tag on Tfb1 (not shown). A silver stain of the peak holoTFIIH fraction can be seen in the results section **Figure 18**. Later, elution fractions were functionally tested for CTD kinase activity and stimulation by the purified Mediator complex. The result of this assay is shown in Figure 19. The peak of TFIIH activity is found in fraction 27.

## 2.11 Biochemical assays and procedures

### 2.11.1 Kinase assay

Assay Conditions (final concentration):

Hepes, pH 7.5	40 mM
MgCl <sub>2</sub>	10 mM
$\beta$ -mercaptoethanol	5 mM
Glycerol	5% (v/v)
$\gamma$ 32P-ATP (3000 Ci/mmol)	10 $\mu$ Ci/20 $\mu$ l
ATP	62.5 $\mu$ M
Polymerase	200 ng/20 $\mu$ l

Kinase assays were usually performed in 20  $\mu$ l final volume. A 2x stock was made of 5x kinase buffer, all nucleotides and fresh  $\beta$ -mercaptoethanol sufficient for 10  $\mu$ l/ reaction. In separate tubes, a 10  $\mu$ l volume of enzymes, substrates and H<sub>2</sub>O were mixed on ice and adjusted with [40 mM Hepes pH 7.5, 500mM KOAc] to equalise the salt concentration between compared fractions ( $\leq$ 100 mM in the final reaction mix). Reactions were started by adding stock to reactions (1:1 volume:volume ) and tubes were incubated at 30°C for 60 min. Reactions were put back on ice and terminated by

adding 5 µl 5x sample buffer and heating to 95°C for 3-5 min. Reactions were analysed by 5% SDS-PAGE and the gel was dried and exposed to film or Phosphorimager plate.

### **2.11.2 *Fcp1* Phosphatase Activity Assay**

Fcp1 phosphatase activity was assayed with RNA polymerase II as a substrate. RNA polymerase II had previously been phosphorylated with TFIIF-kinase. Fcp1 (20-50 ng/reaction) was incubated in a volume of 20 µl under the conditions described below for 30 minutes at 30°C. Reactions were stopped by the addition of SDS loading buffer to 1x. Samples were incubated at 95°C for 3 minutes before loading onto a 5% SDS PAGE gel which was subsequently Coomassie- or silver stained to detect the Rpb1 band.

#### **Assay Conditions**

Tris-Acetate, pH 7.8	40 mM
MgCl <sub>2</sub>	10 mM
DTT	5 mM
Glycerol	20% (v/v)
EDTA	0.1 mM
Tween-20	0.025%
Polymerase	200 ng/20 µl

### **2.11.3 Dissociation assays**

Dissociation assays monitor the treatment-induced appearance in the supernatant of subunits from the non-immobilised part of the holoRNAPII complex. Prior to experiments where coreRNAPII was immobilised on 12CA5 resin, the Protein A part of the TAP tag on Med14 was first removed by purification on IgG resin and elution with TEV protease. This step is necessary to prevent the TAP-tag from binding the constant region of the 12CA5 antibody, effectively immobilising both Mediator and RNAPII subunits.

#### **2.11.4 Preparation of directly coupled affinity beads**

For preparation of directly coupled immuno-affinity beads, monoclonal antibody (anti-HA epitope 12CA5, or anti c-myc epitope 9E10), purified from hybridoma cell supernatant and eluted in PBS, was obtained from the monoclonal antibody service at Cancer Research UK. The antibody (at 1 mg/ml) was concentrated using Centriplus spin concentrators (Centriplus YM-10, Millipore cat. # 4421) until the concentration of 5-10 mg/ml was attained. Activated, cross-linked agarose (Affigel-15, Bio-Rad cat.

#153-6052) was washed into PBS (20 column volumes) before mixing with antibody at a ratio of 1 ml resin to 3 ml antibody and incubating on a roller for 4 hours at 4°C. Unreacted sites were blocked by washing in 100 mM Tris-Cl pH 7.5 and unbound antibody was removed by several washes of PBS. The antibody beads were stored for up to 2 weeks at 4°C before use.

### **2.11.5 Preparation of IgG resin**

For IgG resin preparation, 50 µl bed volume of IgG-Sepharose (GE biosciences, cat #17-0969-01), was dispensed into a 2 ml eppendorf tube and washed sequentially in 2 ml Tris-Saline-Tween (TST): [50mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20] and 2 ml 0.5 M acetic acid (adjusted to pH 3.4 with ammonium acetate) with intermittent pelleting [500 rpm, 2 min, RT, Eppendorf 5415 centrifuge). After two such cycles, the resin was repeatedly washed in batch with TST until the pH of the supernatant was 7.6 (checked with indicator paper). Finally, the resin was equilibrated in 2x 2 ml binding buffer (see below) and the supernatant was removed, leaving the resin just covered.

### **2.11.6 Binding of TAP-tagged holoRNAPII to IgG resin**

The pooled holoRNAPII peak from hydroxyapatite was generally in a concentration of 200-300 ng/µl. For a dissociation assay, 300 µl of this was added to the equilibrated IgG beads and incubated on a roller table for 2 hours at 4°C. Beads were pelleted like above and the supernatant was removed leaving the resin just covered. Unbound protein was removed by washes with 3x 2 ml binding buffer and supernatant was removed as before. If removal of the protein A part of the TAP tag on Med14 was the objective, 300 µl of binding buffer containing 0.5-1 unit/µl TEV protease (Tev protease or AcTev protease, Invitrogen cat # 12575-015) was added to the holoRNAPII bound on the resin and incubated 30 min at 16°C or overnight at 4°C. Eluted holoRNAPII in the supernatant was pooled with 2 further washes of 300 µl binding buffer each.

### **2.11.7 Anti HA-affinity resin binding of holoRNAPII**

Purified holoRNAPII (incorporating Med14-TAP and Rpb8-3HA affinity tagged subunits), was immobilised on either anti HA-affinity resin or IgG resin and TFIID, TFIIF or CTDK-1 was added, with or without ATP to phosphorylate the CTD of RPB1. Beads and supernatants were separated and dissociation was indicated by the ATP-dependent appearance, in the supernatant, of Mediator subunits which was monitored by immunoblotting with antibodies against Med1 or Med4.

## Materials & Methods: Biochemical Assays and Procedures

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The affigel-12CA5 beads were stored at 4°C in PBS. A bed volume corresponding to the number of assays planned (10 µl/assay) was equilibrated by repeated pelleting [500 rpm, RT, Eppendorf 5415 centrifuge] and re-suspension in 3 x 2 ml binding buffer. Supernatant was removed and the pool of holoRNAPII elutions from IgG-sepharose was added and left to bind at 4°C for 2 – 4 hours on a rolling table. Unbound proteins were removed by washes with 2 x 2 ml binding buffer and 2 x 2 ml phosphorylation buffer (see below). Finally, resin was resuspended in a volume 100x the number of dissociation experiments planned and 100 µl aliquots were dispensed in numbered Eppendorf tubes and pelleted as above. From each tube supernatant (80 µl) was removed leaving 10 µl supernatant above beads, and tubes were placed on ice. To each tube, a 10 µl volume of kinases (with or without nucleotides) was added and the reactions were incubated at 30°C for 30-60 minutes before being returned to ice. The supernatants were gently recovered avoiding contact with the beads. Two washing steps of 500 µl binding buffer each were pooled with the initial supernatant sample. Protein in the supernatants were concentrated by extraction with 10 µl Strataclean resin (Stratagene cat # 400714) and 10 µl 2x SDS-PAGE sample buffer was added to each tube containing Strataclean resin or 12CA5-affigel resin. Tubes were heated to 3 min at 95°C and loaded on a 4-12% SDS-PAGE pre-cast gradient gel (4-12% Criterion XT Bis-Tris gel, Bio-Rad cat #345-0124/18 well or # 345-0125/26 well). The gel was run at 200 V constant voltage for approximately 45 minutes and blotted to PVDF-membrane (Immobilon-P, Millipore cat # IPVH00010) as described above.

Supernatants were gently aspirated and both resin and supernatants were analysed by 4-12% SDS-PAGE using pre-cast gels (Bio-Rad XT gel). These pre-cast gels enabled the efficient blotting of the large 200 kDa Rpb1 band while leaving the small 23 kDa Rpb8-3HA band well defined in the gel. The buffers used are described below.

### Phosphorylation buffer

Hepes-HCL pH=7.6 (25°C)	40	mM
BSA	0.1	mg/ml
KOAc	75	mM
Tween20	0.06	%
NP40	0.003	%
MgAc	7.5	mM
Imidazole	3	mM
Glycerol	10	%
B-Mercaptoethanol	5	mM
1x Protease inhibitor cocktail		

### Binding Buffer:

Hepes-HCL pH=7.6 (25°C)	40	mM
BSA	0.25	mg/ml
Tween20	0.2	%
NP40	0.01	%
KOAc	200	mM
Imidazole	15	mM
Glycerol	20	%
β-Mercaptoethanol	5	mM
1x Protease inhibitor cocktail		

HoloRNAPII was immobilised on 50  $\mu$ l bed volume Affigel-12CA5 beads and washed in 4 x 2 ml binding buffer. Binding buffer was exchanged for phosphorylation buffer and holoRNAPII beads were portioned in aliquots of 10  $\mu$ l bed volume. In all reactions, 1  $\mu$ l holoTFIIH (HAP32) was added as well as either H<sub>2</sub>O, the non hydrolysable ATP analogue ATP- $\gamma$ S (1 mM), or ATP (1mM) in total volumes of 30  $\mu$ l. Each reaction was incubated at 30°C 60 min and transferred back to ice. Beads and supernatants were analysed by 4-12% gradient SDS page and the membrane was cut in horizontal strips according to the positions of pre-stained marker bands and subjected to immunoblotting with 12CA5 and Med4 antibodies. The marker used for these blots was normally a broad range prestained marker (Bio-Rad cat. no 161-0373).

### ***2.11.8 Dissociation assay using IgG beads and [ $\gamma$ -<sup>32</sup>P]-ATP***

HoloRNAPII (300  $\mu$ l) was bound to 100  $\mu$ l IgG-sepharose for 2 hours at 4°C and unbound proteins were removed by washing in 3x 2 ml binding buffer as described above. Enzymes were added and salt was adjusted to 100 mM KOAc in a volume of 10  $\mu$ l. The dissociation assay was started by adding 10  $\mu$ l 2x reaction mix containing 200  $\mu$ M ATP and 0.5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P ATP] / $\mu$ l. Tubes were incubated 30 min 30°C. The kinase reaction was stopped by bringing the EDTA concentration to 10 mM with 10  $\mu$ l 30 mM EDTA per reaction. From each tube, 24  $\mu$ l of supernatant was removed for analysis. Phosphorylation buffer was added to make up for the amount removed and beads were sampled by re-suspension by stirring and removal of 10  $\mu$ l bead-slurry for analysis. Sample buffer was added and reactions were analysed by SDS-PAGE. The gel was dried and exposed to phosphorimager plate (Storm 800; Molecular Dynamics). The results were processed and quantified by densitometry using the ImageJ program ([rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)).

### ***2.11.9 Kinase assay to determine linear range for TFIIK and CTDK-1***

Before immobilisation, the protein A part of the TAP tag on Med14 was removed as described in Materials and Methods. The peak of holoRNAPII from the HAP elution profile was purified by binding 300  $\mu$ l HAP fraction to 50  $\mu$ l IgG resin for 2h at 4°C and washing with 2x2ml A300 buffer (see materials and methods) and 3x2ml PBS(see Materials and Methods). Batch elution of HoloRNAPII was achieved by incubation with recombinant TEV overnight at 4°C. Two further batch elutions of 200  $\mu$ l PBS each



## Materials & Methods: Biochemical Assays and Procedures

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were pooled with the first fraction at 4°C. The holoRNAPII concentration was measured by BCA kit (Biorad) to be approximately 0.1 mg/ml and a total of 60 µg holoRNAPII was then applied for 2 hours at 4°C to 300 µl 12CA5-Affigel15 beads pre-equilibrated in kinase buffer. The beads were washed in 3x 2 ml PBS and aliquoted into individual tubes each containing approximately 4 µg holoRNAPII. The supernatant was removed, leaving 10 µl covering the beads. Kinase concentrations were; Tfb3-CPB: 67 ng/µl and Ctk1-CBP: 54 ng/µl. Reactions were initiated by adding 20 µl 2x kinase buffer containing the indicated components to each tube. Final salt concentration was kept at 100 mM KOAc in all experiments.

## 2.12 Primers and strains used

**Table 2 Primers used**

#	Conditions for use	5' – 3' sequence
1	95°C 3 min. then 5 x [95°C 20 sec/55°C 20 sec/72 °C 3 min] then 25 x [95°C 30 sec/Gradient 55°-70°C, 30 sec/72°C, 3 min] and lastly 72°C 10min to finalise products.	GAAATTTGAATAACTTG AAGCAAGAGAACGCTTA TCTTTGATTTCGTCGTTCC GGGTTCTGCTGCTAGT
2	KOD proofreading polymerase	CTAGTAGCAGTAAGTGA TCGCCCTTTTTTTTAGCA CTCGGTCCAATGCTGCCT CGAGGCCAGAAGAC
3	94°C 3 min. then 10 x [94°C 10 sec/55°C 1min /72 °C 1 min] then	GGATCCATGGTGAGCAA GGGCGAGGA
4	25 x [94°C 20 sec/Gradient 52°-65°C, 30 sec /72°C, 2 min] and lastly 72°C 10 min to finalise products. KOD proofreading polymerase	CTTGTACAGCTCGTCCAT GC
5	94°C 3 min. then 10 x [94°C 10 sec/55°C 1min /72 °C 1 min] then 25 x [94°C 20 sec/Gradient 55°C-70°C , 30 sec /72°C, 2 min]	GCATGGACGAGCTGTAC AAGCTGCAAGTTTCTCC AACTTCCCCAAC
6	and lastly 72°C 10 min to finalise products. KOD proofreading polymerase	ACAACAGCCTCCAGCAC CAGTTCTTCTGGAATTTT CATTTTCA
7	94°C 3 min. then 10 x [94°C 10 sec/55°C 1min /72 °C 1 min] then	CACCGGATCCATGGTGA GCAAGGG
8	25 x [94°C 20 sec/Gradient 55°C-70°C , 30 sec /72°C, 2 min] and lastly 72°C 10 min to finalise products. KOD proofreading polymerase	GAATTCTTATCACTAAC CTCCACCACAACACCCT GGACAACAGCCTCCAGC ACCAG
9	95°C 3 min. then 5 x [95°C 20 sec/55°C 20 sec /72 °C 3 min] then 25 x [95°C 30 sec/61°C , 30 sec /72°C,3 min] and lastly 72°C 10 min to finalise products. KOD proofreading polymerase	ATTCTCCAAAGCAAGAC GAACAAAAGCATAATGA AAATGAAAATTCCAGAG CTGAAGAGCAAAAATA ATTTCTGAAGAAGATT ATTGTGCTGTCCAGGTTG TTGTTAACCTCGAGGCC AGAAGAC
10		AAACTATATATAATGTA ATAACGTCAAATACGTA AGGATGATATACTATAT CCGGTTCTGCTGCTAGT
11	94°C 3 min. then 5 x [95°C 20 sec/55°C 20 sec /72 °C 3 min] then	CACCATGGCTGACCAAC TGACA
12	25 x [95°C 30 sec/61°C , 30 sec /72°C,3 min] and lastly 72°C 10 min to finalise products. KOD proofreading polymerase	CTTTGCTGTCATCATTTG TAC
13	Sequencing primer. 30 cycles [94°C 30 sec/ 55 °C 30 sec /60 °C 4 min]	GTAAAACGACGGCCAG

## Materials & Methods: Primers and Strains Used

**Table 3 Strains used**

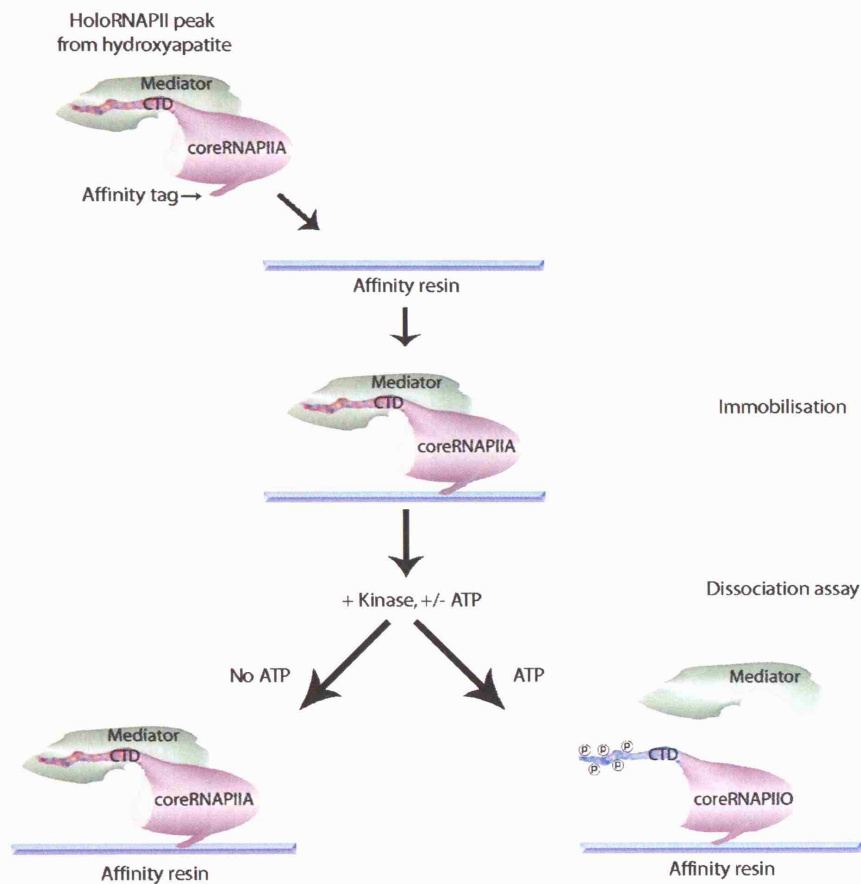
Number	Description	Genotype
1	Ctk1-TAP	<i>S288C(ATTC 201388: Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) Ctk1::Ctk1-TAP-HIS3MX6)</i>
2	Med14(Rgr1)-TAP	<i>CB101, Mata, pep4::HIS3/prb1::LEU2, prc1::HISG, can1, ade2, trp1, ura3, his3, leu2-3, 112, Rgr1::Rgr1-TAP-Trp (Borggreffe et al., 2001)</i>
3	Med8-TAP	<i>CB101, Mata, pep4::HIS3/prb1::LEU2, prc1::HISG, can1, ade2, trp1, ura3, his3, leu2-3, 112, Med8::Med8-TAP-Trp (Borggreffe et al., 2001)</i>
4	Tfb3-TAP	<i>CB101, Mata, pep4::HIS3/prb1::LEU2, prc1::HISG, can1, ade2, trp1, ura3, his3, leu2-3, 112, Tfb3::Tfb3-TAP-Trp (Borggreffe et al., 2001)</i>
5	Rgr1-TAP, Rpb8-3HA	<i>CB010, Mata, pep4::HIS3/prb1::LEU2, prc1::HISG, can1, ade2, trp1, ura3, his3, leu2-3, 112, Rgr1::Rgr1-TAP-Trp, Rpb8::Rpb8-3HA-Ade (this study)</i>
6	Rpb1--HA-2xXa-CTD	<i>Z26, [a ura3-52 leu2-3 leu2-112 his3-A300 rpb1-187::HIS3/pRP112 (URA3 CEN4 RPBI), Rpb1-HA-2X2-CTD encoded by plasmid YCpL14 (Li and Kornberg, 1994)</i>
7	JSY787 <i>elp1Δelp4Δ</i>	<i>W303, ura3 leu2-3, 112 his3-11,15 ade2-1 can1-100 ELP1::LEU ELP4::ADE, mating type α (Gift from Thodoris Petrakis)</i>
8	JSY556 Rpb2-HIS-HA W303	<i>W303-1a. ura3 leu2-3, 112 his3-11,15 ade2-1 can1-100. mating type A, His-HA-Rpb2::TRP in (Svejstrup lab).</i>

The work described below was carried out in the laboratory of Dr. Jesper Svejstrup. I have divided it into two parts. Part I describes my efforts to answer the initial question of whether CTD hyperphosphorylation can cause dissociation of holoRNAPII. Part II contains a description of ongoing work done with a view to further extend on the observations in part I.

### ***Part I***

### **3. Results**

Events taking place during promoter clearance or, – the initiation-elongation transition – remain poorly understood. However, it is thought that dissociation of holoRNAPII into Mediator and core RNAPII occurs at this point and that this event coincides with a transition from hypo- to hyper-phosphorylation of the RNAPII CTD. Indeed, the Mediator complex has been shown to only be associated with RNAPII when it is at the promoter region (Svejstrup et al., 1997). Conversely, the Elongator complex has been shown to be associated with elongating RNAPII on chromatin in a salt-stable manner (Otero et al., 1999). The question posed at the outset of the project was whether CTD phosphorylation per se is sufficient to disrupt the connection between Mediator and coreRNAPII. Moreover, if phosphorylation itself was not sufficient, the possibility that the Elongator complex might be required to stimulate dissociation through competition with Mediator for occupancy of the hyperphosphorylated CTD was to be investigated. Finally, other general questions about CTD phosphorylation and its effect on CTD tertiary structure were to be investigated. I chose to investigate these problems using highly purified proteins in defined biochemical assay systems. The initial experimental design was to purify holoRNAPII from a yeast strain expressing an epitope-tagged version of one of the RNAPII subunits. Then, purified holoRNAPII would be immobilized on an affinity resin and a dissociation assay would be preformed



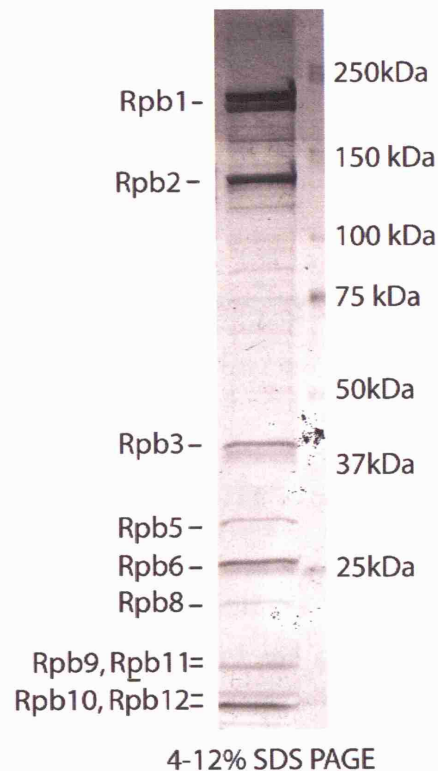
**Figure 9 Illustration of the holoRNAPII dissociation assay**

The procedure entailed adding highly purified holoTFIIH (with or without ATP) to phosphorylate the CTD of Rpb1. The ATP-dependent appearance in the supernatant of Mediator specific subunits (identified by immunoblotting) would indicate that phosphorylation of the CTD causes holoRNAPII dissociation.

Purification of coreRNAPII, holoRNAPII, holoTFIIH and other proteins was necessary to establish this system. Fractions containing HoloRNAPII and holoTFIIH can be derived from the hydroxyapatite step in the purification procedure described by (Kim et al., 1994). To assay the hydroxyapatite fractions for CTD kinase activity, a CTD kinase assay was established, which used homogeneous coreRNAPII as substrate. The following pages will first describe the purification of these protein complexes and then their use in the dissociation assay.

### 3.1 Purification of coreRNAPII

The purification of coreRNAPII was done by the procedure described (Edwards et al., 1990) and details of the procedure can be found in Materials and Methods. A silver stained SDS-PAGE analysis of the final product is shown in Figure 10. The concentration of the protein was determined by Bradford assay to be 0.3 mg/ml.



**Figure 10 Purified CoreRNAPII**

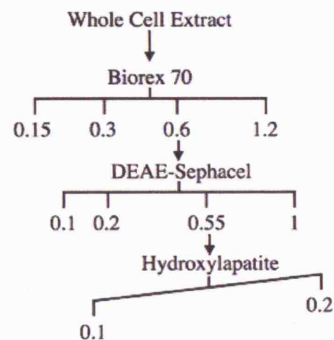
Coomassie stain of core RNAPII purified from yeast extract using Heparin, 8WG16 immuno-purification and DEAE ion exchange steps. The subunits have been assigned identities according to molecular weight (Sayre et al., 1992a).

### 3.2 Purification of (Rpb2HisHa) Holopolymerase

Holopolymerase subunits Rpb4 and Rpb7 are dissociable from the RNAPII complex giving rise to two different forms of RNAPII. While the two RNAPII forms have equal processivity *in vitro*, only the form encompassing both Rpb4 and Rpb7 is competent for promoter directed transcription initiation (Edwards et al., 1991). The Rpb4/7 module is sometimes substoichiometric in polymerases purified from log phase cells, whereas RNAPII purified from cells in late-log to early stationary phase is a 12 subunit stoichiometric complex (Choder and Young, 1993). Hence, yeast strains for

## Results Part I

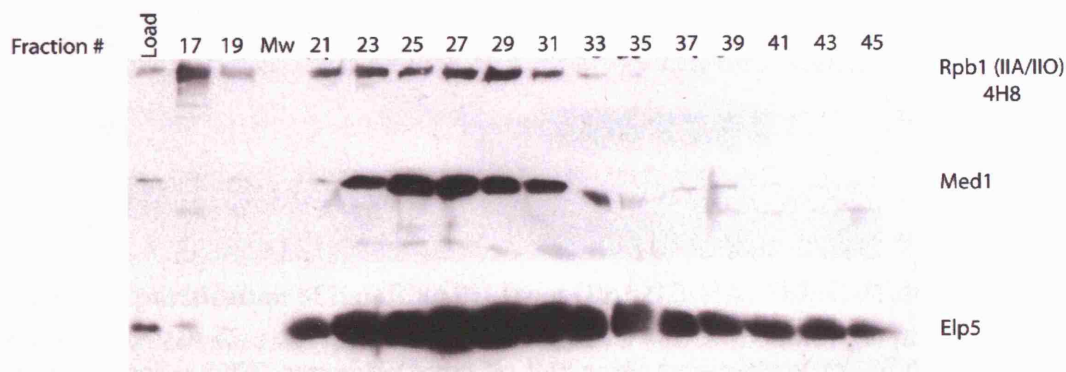
holoRNAPII purification were grown to early stationary phase before harvesting. The purification of holopolymerase was done according to a 5-column procedure first described by (Kim et al., 1994) Figure 11. The hydroxyapatite step in the procedure separates the basal transcription factors from core Mediator and RNAPII holoenzyme. Details of the procedure can be found in Materials and Methods.



**Figure 11** The procedure used to purify GTF's, coreRNAPII and holoRNAPII

Adapted from (Kim et al., 1994).

An immunoblot across the elution profile from the HAP column is shown in Figure 12. A peak of RNAPII is visible in fraction 17 and a separate peak of holoRNAPII co-eluting with the Med1 subunit of the Mediator complex indicates elution of holoRNAPII (fractions 21-31), in agreement with the published behaviour of holoRNAPII at this stage in the purification (Kim et al., 1994). Interestingly, the Elp5 subunit from the small sub-complex of the Elongator complex was also detectable in all holoRNAPII containing fractions.



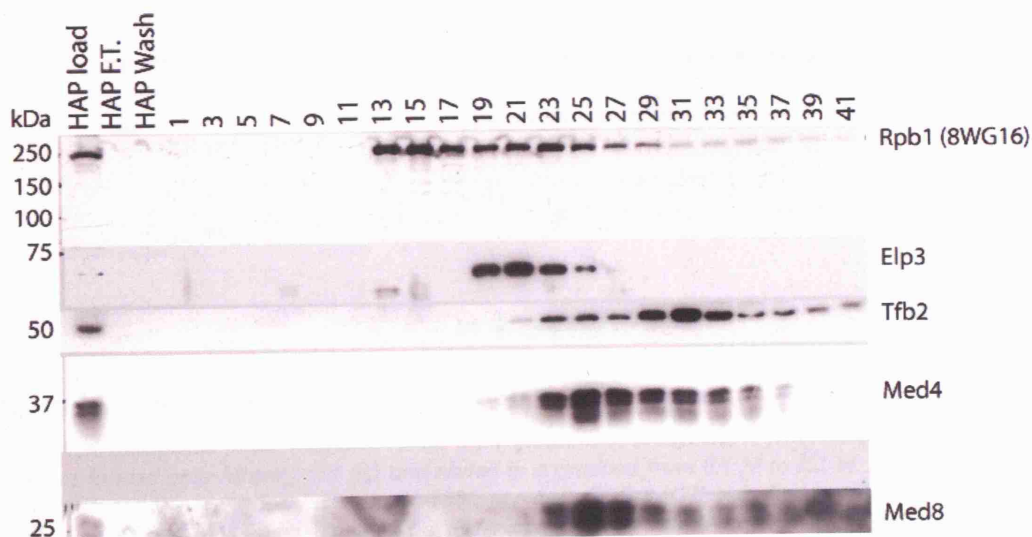
**Figure 12** Immunoblot across HAP elution profile.

*HoloRNAPII purification from Rpb2HisHA. Every second fraction in the HAP elution profile was analysed by 10% SDS-PAGE and proteins were blotted to a PVDF membrane for immunodetection using the designated antibodies.*



### 3.3 Purification of holoRNAPII devoid of Elongator complex

One of the original objectives of the project was to investigate whether the Elongator complex might be required to effect the dissociation of Mediator and RNAPII. As demonstrated by immunoblot (see Figure 12) every HAP fraction containing holoRNAPII also contained the Elp5 subunit belonging to the small Elongator sub-complex. Furthermore, the large sub-complex of Elongator has previously been shown to co-elute with RNAPII on MonoQ during a purification procedure similar to the one employed here (Li et al., 2001). At this early stage of the project, I was of the opinion that an Elongator-free preparation of holoRNAPII was necessary to set up an assay for holoRNAPII dissociation. To achieve an Elongator-free holoRNAPII, two yeast strains (# 7 & 8 - see details in strains table in Materials and Methods) were mated: one expressing a genomically tagged Rpb2-HisHA subunit and other lacking the *ELP1* and *ELP4* genes. The growth rate of the new *Rpb2-HisHA elp1Δ elp4Δ* strain was equal to the *elp1Δ elp4Δ* parental strain, indicating that the HisHA tag on Rpb2 did not adversely affect yeast growth. 100 litres of this yeast strain was grown in batch fermentation, lysed by bead beating and the supernatant was cleared from debris by centrifugation and frozen in liquid nitrogen (lq. N<sub>2</sub>). HoloRNAPII was purified as described above. An immunoblot across the HAP fraction elution profile is shown in Figure 13

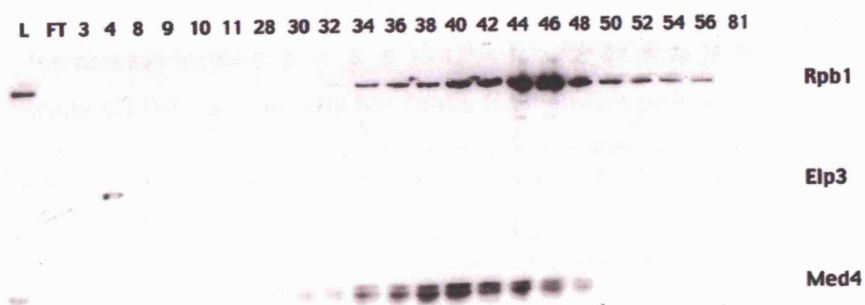


**Figure 13 purification of holoRNAPII from (Rpb2HisHA,  $\Delta$ Elp1,-4) strain**

The indicated HAP fractions (5  $\mu$ l/lane) were separated on a 4-12 % SDS-PAGE gel (Bio-Rad precast), and transferred to PVDF. Immunoblot across the HAP profile for subunits of RNAPII (Rpb1), Mediator (Med4), TFIIF (Tfb2) and Elongator (Elp3) was performed. CoreRNAPII peaks in fraction 15 (8WG16), while holoRNAPII (indicated by Med4 & Med8) peaks in fraction 25. Both Med4 and Med8 are modified by phosphorylation and appear as doublets (Balciunas et al., 2003). A component of the large Elongator sub-complex (Elp3) peaks in fraction 21 and is visible across the holoRNAPII containing fractions. The TFIIF subunit Tfb2 peaks in fraction 31



Despite lacking the *ELP1* and *ELP4* gene products, the Elp3 protein was still detectable and seemed to elute from the HAP column between coreRNAPII and holoRNAPII (fractions 19 -23) with a tail stretching into the holoRNAPII peak. To remove all traces of Elp3 and to further purify holoRNAPII, the peak fractions (23 -27) were pooled and loaded directly onto a MonoQ HR 5/5 (Pharmacia) column equilibrated in 100 mM KOAc and eluted with a gradient of 100 mM KOAc to 1.2 M KOAc (Myers et al., 1998). An immunoblot of the resulting MonoQ elution profile is shown in Figure 14. In this profile, the peak of Mediator can be seen in fractions 38-40 (evidenced by the Med4 signal peaking around 600 mM KOAc) whereas Rpb1 peaks later in the profile at fractions 44-46 (corresponding to 800 mM KOAc). Elp3 eluted much earlier (fraction 4). This behaviour of RNAPII and Mediator is in good agreement with (Myers et al., 1998) who found the majority of Rpb1 in the MonoQ elution profile to be part of holoRNAPII (peaking in fractions 44-46), which indeed peaked later than did the 'free' Mediator complex (the majority of Mediator peaking in fractions 38-40). The fractions containing the holoRNAPII peak (fractions 44-47) were pooled and snap frozen in liquid N<sub>2</sub>. The protein concentration of the pooled fractions was 234 ug/ml, measured using a Bio-Rad kit.



**Figure 14 Immunoblot of MonoQ elution profile**

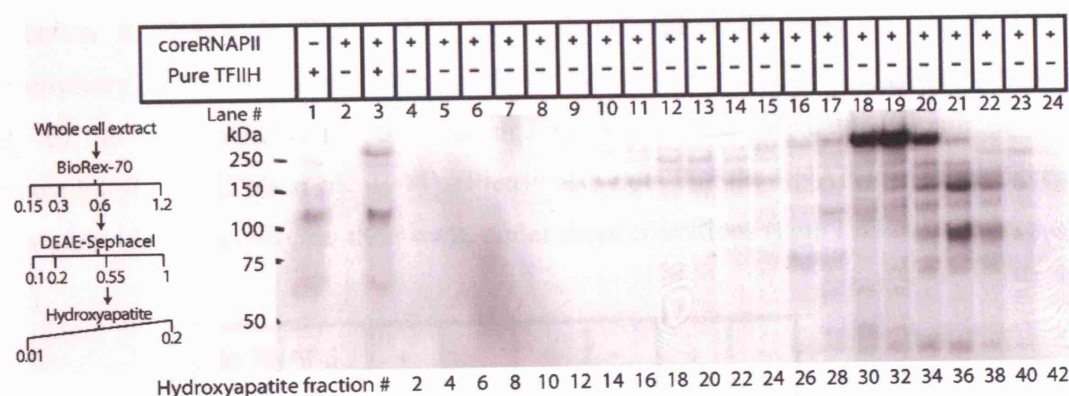
*Pooled hydroxyapatite peak fractions, from a purification of holoRNAPII from an *elp1Δ elp4Δ* strain of yeast was loaded onto MonoQ HR 5/5 and eluted in a gradient from 0.1 M to 1.2 M. 5μl from each of the indicated fractions were loaded onto a 10% pre-cast SDS-PAGE gel. Immunoblotting for RNAPIIa (8GW16), Elp3 and Med4 demonstrated the absence of Elp3 in holoRNAPII fractions (fr. 44 – 47).*

### 3.4 Purification of highly active TFIID

The procedure used to purify holoRNAPII is identical to the one developed to purify all of the components needed for the basal transcription machinery (Sayre et al., 1992b).

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This proved useful in the purification of TFIIF, as this basal transcription factor elutes near the end of the phosphate gradient applied to develop the third column of the procedure; the hydroxyapatite (HAP) column (Kim et al., 1994; Sayre and Kornberg, 1993). To identify fractions containing CTD specific kinase activity, purified coreRNAPII (Figure 10) was used as a substrate in a kinase assay across the HAP elution profile. A limited amount of holoTFIIF previously purified by Jesper Svejstrup was available for use as a positive control for CTD kinase activity. The autoradiogram of the kinase elution profile is shown in Figure 15. As expected (Sayre et al., 1992a), the peak of CTD kinase activity resides in fractions 30-34, at the end of the 0.01 M to 0.2 M gradient of inorganic phosphate. Other experiments showed that the phosphorylated 250 kDa band was indeed Rpb1, as expected (see below).



**Figure 15 Strong CTD-kinase activity late in the HAP elution profile**

*Autoradiogram of a 10 % SDS-PAGE analysis of a kinase assay, testing the CTD-kinase activity of HAP column fractions. For each reaction, 5 $\mu$ l HAP fraction was used with purified coreRNAPII as the substrate. The purification procedure is outlined on the left. TFIIF purified by J. Svejstrup served as a positive control (lane 3). The details of the procedure as described in Materials & Methods. Specific CTD kinase activity is evident in fractions 30-34. The run was done at 100 mM KOAc with a 0.01M to 0.2 M gradient of potassium phosphate.*

### 4.3.1 Peak of CTD activity co-elutes with TFIIF subunit Tfb2

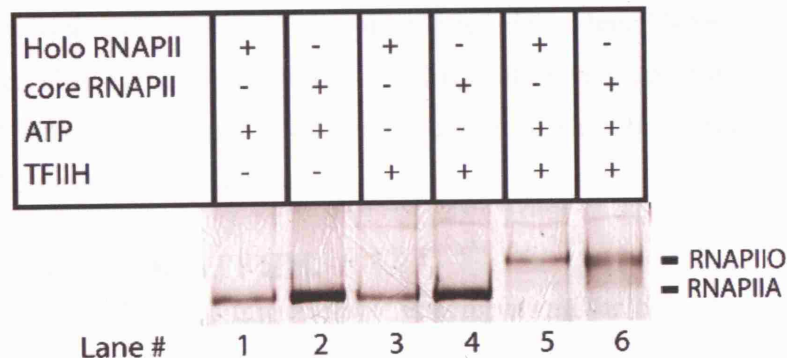
The autoradiogram in Figure 15 shows a peak of strong CTD specific kinase activity in fraction number 32. Immunoblotting using anti-Tfb2, anti-Ccl1 and anti-Kin28 antibodies shows co-elution of TFIIF specific subunits in fraction 32, indicating that TFIIF is indeed the kinase responsible (see Figure 13, fraction 29-33). Other protein species co-elute with the peak of TFIIF from HAP, resulting in the preparation only being partially pure. Unfortunately, attempts to purify holoTFIIF through further chromatography of the pooled peak fractions on MonoS or MonoQ resulted in a

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complete loss of CTD kinase activity. The fragility of the TFIIH complex has been noted by others (Svejstrup et al., 1995) and this is probably the reason for the loss of activity.

### 3.4.2 Hyperphosphorylation of Rpb1 by holoTFIIH

The peak of CTD kinase activity was found in HAP fraction 32. To confirm that these fractions did indeed hyper-phosphorylate RNA polymerase II and to characterize further the phosphorylation of RNAPII, a kinase assay using radioactive [ $\gamma$ - $^{32}$ P]-ATP was performed, using HAP fraction 32 to phosphorylate coreRNAPII and holoRNAPII in conjunction with two visualization procedures. The reactions were analysed on 5% SDS-PAGE which was silver stained to detect the phosphorylation-dependent bandshift of the large, CTD-containing Rpb1 subunit (Figure 16). An autoradiogram of the reaction is shown in Figure 17. Note that holoRNAPII was completely hyperphosphorylated in this assay, whereas coreRNAPII was not (Figure 16, compare lane 5 & 6), in agreement with the finding by others that Mediator stimulates CTD phosphorylation (Kim et al., 1994). Please also note that the RNAPII fractions have no apparent kinase activity on their own, under these conditions (lane 1 & 2).



**Figure 16** Hyperphosphorylation holoRNAPII by TFIIH

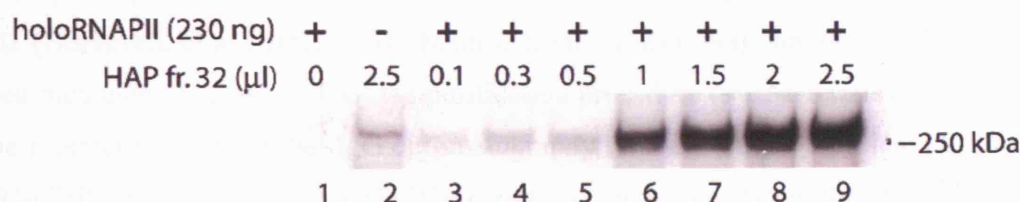
A silver stained 5% SDS-PAGE analysis of a kinase assay using 1  $\mu$ l TFIIH/HAP f. 32, 1  $\mu$ l coreRNAPII (100 ng/ $\mu$ l) or holoRNAPII (230 ng/ $\mu$ l) pr assay. Where indicated, 0.6 mM ATP was added. Buffer conditions were as described in Materials and Methods. The mobility shift induced by TFIIH phosphorylation is quantitative in the case of holoRNAPII where the addition of 1  $\mu$ l TFIIH was sufficient to completely shift the Rpb1 band to the IIO position.

The data in Figure 16 show that the highly active TFIIH-containing HAP fraction is able to phosphorylate the Rpb1 subunit of holoRNAPII and that hyperphosphorylation of the Rpb1 CTD causes a retardation of migration in a 5% polyacrylamide gel, giving rise to



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a mobility gel-shift of the IIO form (lane 5 & 6). The amount of TFIIH added to achieve this band-shift was 1  $\mu$ l, corresponding to lane 6 in Figure 17.



**Figure 17 Phosphorylation of holoRNAPII**

*Autoradiogram of a 10% SDS-PAGE analysis of a kinase reaction. CTD kinase activity is detected in the assay using HAP fraction 32 to phosphorylate holoRNAPII from a MonoQ peak. Phosphorylation of 230 ng holoRNAPII reaches saturation at 2  $\mu$ l HAP fraction 32. Assay done at 600  $\mu$ M ATP and 100 mM KOAc. Reactions were incubated at 30°C 30 min. As can be seen from lane 2, HAP fraction 32 contains trace amounts of polymerase.*

Interestingly, from comparing lane 6 with lanes 7 to 9 in Figure 17, it is clear that despite a quantitative shift of RPB1 to the IIO position (see Figure 16), the radioactivity of the Rpb1 band still continues to increase as more TFIIH is added, indicating that the phosphorylation is not exhaustive at 1  $\mu$ l added holoTFIIH (HAP f. 32). This result from experiments with yeast RNAPII supports the idea that not every single CTD repeat needs to be phosphorylated for the phosphorylation-dependent RNAPII mobility change to take place. This agrees well with the findings of (Zhang and Corden, 1991b) who showed that only 15-20 phosphates were required to shift the mammalian CTD (52 repeats) to the slower mobility form.

### 3.5 Purification of holoTFIIH.

HoloTFIIH is a fragile 9 subunit complex encompassing the helicase subunit Ssl2 and its purification to homogeneity by using 4 steps of conventional chromatography combined with an affinity purification step utilizing a 6xHis epitope tag on the Tfb1 subunit was previously described in (Svejstrup et al., 1994). In my (substantially different) procedure, the first three chromatographic steps resulted in a very active CTD kinase preparation. Unfortunately, the highly active HAP fraction also contained other proteins, amongst them traces of Mediator and RNAPII, which might potentially obscure the detection of Mediator subunits dissociating from immobilised holoRNAPII. Moreover, the contaminating proteins might interfere with the dissociation mechanism per se. Thus, there appeared to be a need to obtain purer TFIIH. As mentioned above,

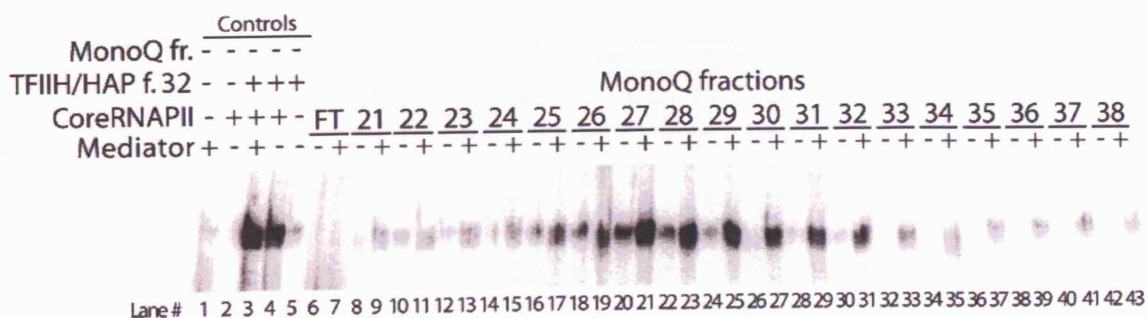
## Results Part I

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attempts at further purification of the TFI<sub>II</sub>H peak on MonoS or MonoQ resulted in substantial loss of kinase activity (data not shown), probably due to the complex falling apart upon further purification. To selectively purify a holoTFI<sub>II</sub>H complex encompassing the helicase subunit Ssl2, a strain expressing a TAP-tagged version of Ssl2 (Borggrefe et al., 2001) was obtained from the Kornberg lab and purification was attempted using the gentle TAP tag purification procedure (see Materials and Methods). The product from the initial IgG chromatography step was only a faint band the size of Ssl2-CBP and no active holoTFI<sub>II</sub>H could be recovered using this procedure (not shown). An alternative purification, using the procedure described for holoRNAPII, followed by immunoblotting for Tfb3 and Ssl2 to identify the holoTFI<sub>II</sub>H peak and finally IgG chromatography of these fractions to pull down holoTFI<sub>II</sub>H also failed to recover active holoTFI<sub>II</sub>H (not shown). Preferential loss of the Ssl2 subunit during TFI<sub>II</sub>H purification has been noted previously (Wang et al., 1994) as has the dynamic association of Ssl2 with coreTFI<sub>II</sub>H (Feaver et al., 1993). Taken together, these factors probably caused the Ssl2-TAP-based strategies to fail. Consequently, I decided to purify TFI<sub>II</sub>H from a strain of yeast expressing a 6xHis tagged version of Tfb1, according to the published protocol (Svejstrup et al., 1994). This procedure consists of two steps of ion exchange chromatography (BioRex70 and Phosphocellulose) followed by an affinity chromatography step using the 6 histidine tag on TFB1-6xHis to pull down TFI<sub>II</sub>H. Finally, the pooled elution fractions from NiNTA are concentrated and further purified using a MonoQ strong anion exchange column. The procedure is described in details in Materials and Methods. The elution of TFI<sub>II</sub>H from the MonoQ column, was detected by immunoblot using antibodies recognising the CTD kinase subunit Ccl1 and antibodies against the 6xHis tag on Tfb1 to identify peak holoTFI<sub>II</sub>H fractions (not shown) and a silver stained SDS-PAGE analysis of the holoTFI<sub>II</sub>H peak fraction from the final MonoQ column is shown in Figure 18.

*Silver stained 10% SDS-PAGE analysis of peak fraction of holoTFIIH from MonoQ HR 5/5. The identity of the bands have been designated based upon the published gels (Svejstrup et al., 1994; Takagi et al., 2003). The concentration of holoTFIIH was 20 ng/μl approximately.*

As can be seen the holoTFIIH complex is very pure, with few contaminating bands. When purified Mediator later became available (see Figure 39) a kinase assay was set up to test functionally, the ability of Mediator to stimulate TFIIH kinase activity. The figure is shown in Figure 19. The peak of activity corresponded to the previously determined peak.



A kinase assay was used to identify fractions containing CTD kinase. The stimulation by presence of the Mediator complex (Kim et al., 1994) was used to identify fractions containing holoTFIIH. Fraction 27 (lane 21 and 21) contains the peak of holoRNAPII. Assay done in 100mM KOAc and 1 mM ATP. Where indicated 1 $\mu$ l TFIH/HAP f. 32 or 1  $\mu$ l purified Mediator (50 ng/ $\mu$ l) was added. RNAPII was 200 ng in each reaction.

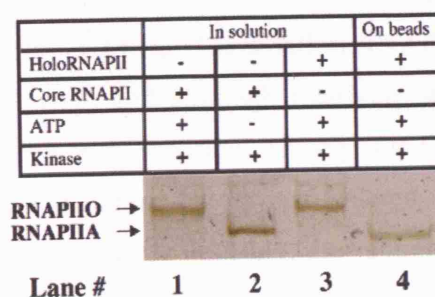
## Results Part I

This peak fraction, or fractions derived in a similar way, was typically used for the assays described below.

### 3.6 RNAPIIA and IIO forms

### 3.7 Immobilisation inhibits hyperphosphorylation

The purified TFIIFH fractions I had obtained were able to hyperphosphorylate both coreRNAPII and holoRNAPII, and the hyperphosphorylated form (designated RNAPIIO) exhibited a mobility shift compared to the hypo-phosphorylated form (IIA). With these active reagents in hand, I now immobilized RNAPII on 12CA5 antibody beads via the tag on Rpb2 to initiate work on the dissociation assay. Surprisingly, immobilisation of holoRNAPII on 12CA5 antibody conjugated Protein A sepharose beads abolished phosphorylation of RNAPII (Figure 20, lane 4). To counter any possible steric hindrance effects it was attempted to immobilise holoRNAPII using the 6xHis-part of the tag to immobilise holoRNAPII on NiNTA resin (Qiagen Superflow) in order to use the HA-part as a spacer, but the inhibition was unchanged. Strangely, even after optimising the phosphorylation reaction conditions (enzyme concentration and enzyme purification batch, salt concentration, and buffer composition) I failed to achieve quantitative hyperphosphorylation of RNAPII immobilized this way. The inhibition of hyperphosphorylation of the immobilised holopolymerase may be due to a steric hindrance effect, as the position of the HisHa tag on the C-terminal of Rpb2 turns out to be very close to both the “attachment point” of the linker which links the CTD to RNAPII as well as location of the Rpb4/Rpb7 sub-module (See Figure 21).



**Figure 20** Inhibition of phosphorylation of immobilised RNAPII.

Silver stained 5% SDS-PAGE analysis of a CTD kinase reaction. Comparison of free vs. immobilised RNAPII when phosphorylated with TFIIFH from HAP fraction 32 (TFIIFH/HAP f. 32). Immobilisation is inhibitory to quantitative hyperphosphorylation. The result was identical using the very pure holoTFIIFH peak fraction (not shown).

This area of RNAPII is probably fraught with important protein-protein and protein-RNA interactions during the initiation-elongation transition. Two different RNA exit paths have been proposed based on groves of positive charge running on the surface of RNAPII. One path goes down toward Rpb4/Rpb7 and the other runs across RNAPII, toward Rpb8 (Gnatt et al., 2001; Westover et al., 2004). The human splicing factor U2AF65 was reported to be associated with RNAPII at initiation, but to immediately change location to interact with nascent mRNA when this emerges from the mRNA exit channel (Ujvari and Luse, 2004) implying that U2AF65 is closely associated with this area or even the CTD. Interestingly, recent photo-crosslinking studies of the exit path of RNA suggest that Rpb7 is contacted by nascent RNA up to a transcription length of 32 nucleotides and in addition, that the contact is lost when the RNA reaches 125 nucleotides (Ujvari and Luse, 2006). With these mechanisms in mind, it is conceivable that Rpb4/Rpb7 could in some way coordinate the CTD as well. As all other efforts had failed, I now decided to try to solve the situation by placing the tag somewhere else than Rpb2.

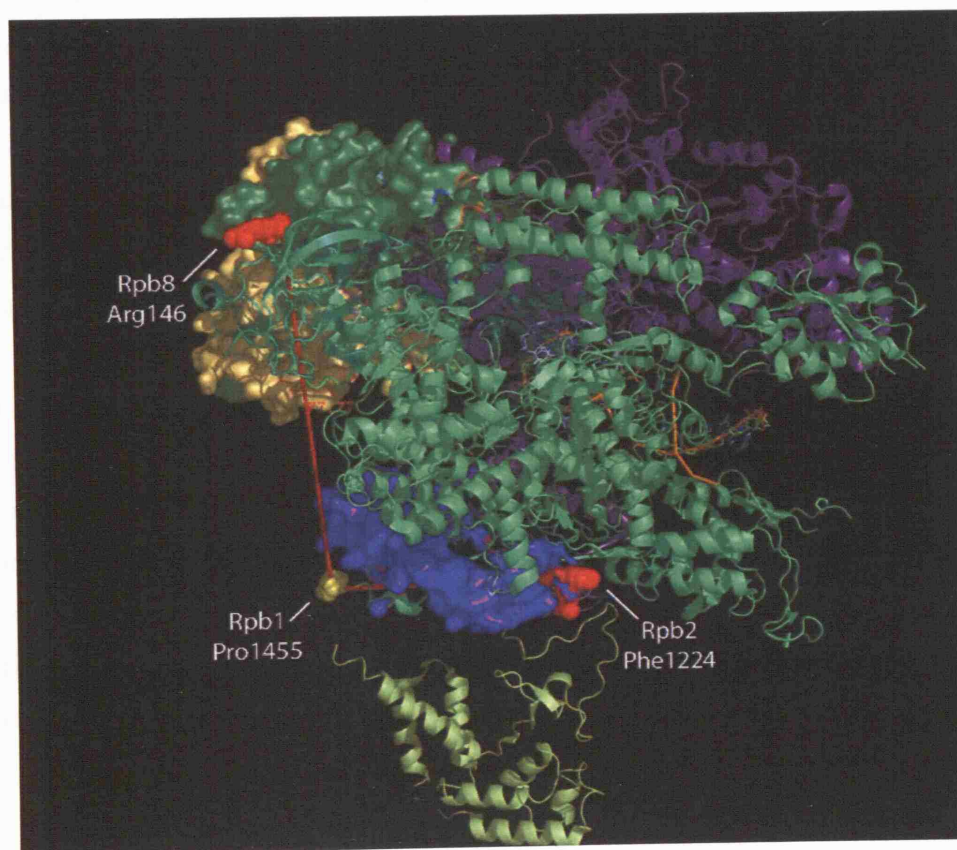
### **3.8 Construction of a Med14-TAP, Rpb8-3HA strain**

In order to overcome the problems with phosphorylation of Rpb2-immobilized RNAPII, a new, more optimal location for the affinity tag was identified according to a number of criteria. Firstly, a C-terminal affinity tag sequence was preferred, because its introduction could be achieved by straight-forward recombination into the yeast genomic site. Secondly, the size of the tag should be small compared to its target. The triple HemAgglutinin (3xHA)-tag is a (YPYDVPDYA)<sub>3</sub> sequence derived from a protein on the surface of human influenza virus (Wilson et al., 1984). The 3xHA epitope has a predicted Mw of a only ~3.2 kDa. The smallest RNAPII subunits are less than 10 kDa in size (Rpb10 and Rpb12) and although relatively small, such a triple HA tag might sterically interfere with their function. Thirdly, the C-terminus of the candidate should be solvent-exposed (as judged by its last ordered amino acid in the RNAPII crystal structure (Armache et al., 2005) Fourthly, the subunit should not to be known to interfere with specific aspects of RNAPII life – such as in the stress response (Rpb4 and Rpb7)(Choder and Young, 1993), or in DNA interaction (Rpb5 and Rpb9 – part of the DNA pinching “jaw structure” and Rpb6 – part of the “Clamp” structure) (Cramer et al., 2000), in order to avoid possible perturbation of the essential functions of the RNAPII



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complex. Finally, subunits known to (or likely to) interact with Mediator were avoided, so as not to risk any steric hindrance effects, in the context of holoRNAPII. The location of these subunits (Rpb3, -6, -11, and -12) are described in the introduction. Taking all these factors into account, I chose to tag the Rpb8 subunit. Rpb8 was tagged by transformation of a PCR product as described in materials and methods. The distance between the last ordered residue of Rpb1 and the last ordered residues of Rpb2 and Rpb8 is illustrated in Figure 21 which is based on the 12 subunit RNAPII crystal structure, PDB: 1Y1W (Kettenberger et al., 2004). By changing the position of the tag, the distance from tag to the base of the CTD was increased from 41.2Å to 77.7Å.



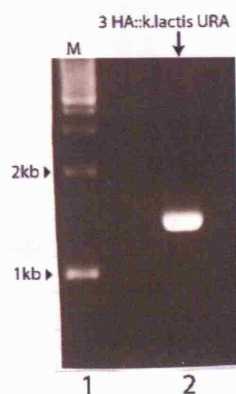
**Figure 21 A side view of coreRNAPII**

*Drawing based on the RNAPII crystal structure (1Y1W), illustrating the location of the last ordered residues of Rpb1, 2 and 8 (see labels). Rpb7, -5, -9 have been hidden from view for clarity. The subunits which interact with Mediator are shown in surface representation (Davis et al., 2002). The red bars between the C-termini, measure the direct distance between the C-terminals of Rpb1 and Rpb2 (41.2Å) and Rpb1 and Rpb8 (77.7Å). Illustration made in the program Pymol (Delano Scientific LLC).*

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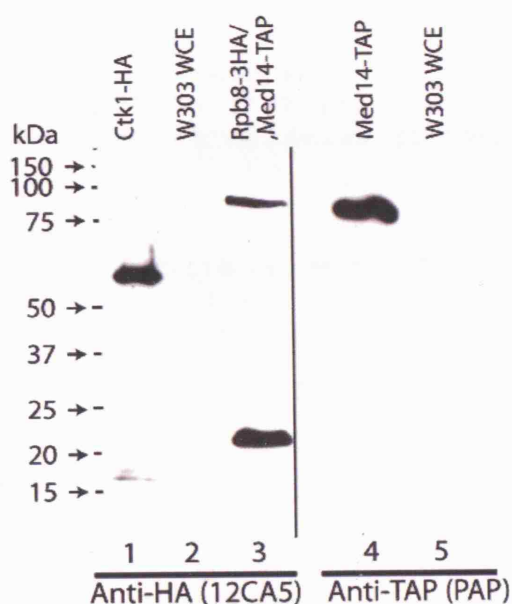
Besides being able to detect dissociation of Mediator from immobilized RNAPII, it was also desirable to be able to detect dissociation of RNAPII from immobilized Mediator. To achieve this, it was necessary also to tag a Mediator subunit. The Mediator complex had previously been successfully purified using a 3xFLAG affinity tag on the C-terminus of the Med14 (Rgr1) subunit of the Middle/head Mediator sub-module (Liu et al., 2001), indicating that this subunit was functionally amenable to tagging. Furthermore, the Med14 (Rgr1) subunit is specific to the large form of Mediator and absent from a smaller Medc complex that has also been identified in crude extracts (Liu et al., 2001). To eventually be able to combine both ways of performing the dissociation assay using the same holoRNAPII preparation, a Med14-TAP strain was procured from the Kornberg lab (strain #2 see Materials and Methods) and used as the basis for 3xHA tagging of Rpb8, enabling the purification of a doubly affinity tagged holoRNAPII (*RPB8-3HA MED14-TAP*). Other experiments had shown that the Elongator contamination of holoRNAPII fractions observed early on was not a problem once the holoenzyme was affinity-purified (bound to beads and washed carefully), so deletion of *ELP* genes was fortunately not necessary. Moreover, as an added advantage, the Rpb8-3HA tag would serve to identify coreRNAPII dissociating from beads and set it apart from the small amount of coreRNAPII potentially present in other protein preparations used in the assay. The Rpb8 tagging was achieved by transformation of the *MED14-TAP* strain with a PCR product with 50 nucleotide homology to the end of the coding region (deleting the original stop codon, and enabling the insertion of a tag and a new stop codon), and the 3' un-transcribed region, respectively, of the Rpb8 gene, as described in Materials and Methods. The product was purified on spin columns and 5µl analysed on a 1.2% agarose gel (see Figure 22).



**Figure 22 Analysis of Rpb8 epitope tagging PCR product.**

*DNA in the 1.2% agarose gel was stained by adding 0.5 mg/litre ethidium bromide to the melted agarose solution. The *k.lactis* URA3 gene comprises 1167 bp (genbank accession Y00454) and together with the homologous sequences on the oligo's as well as the tag sequences itself; a product of around 1400pb is expected for the 3HA::URA tagging product. The size of the bands corresponds to the expected size for the *k.lactis* URA marker.*

The DNA was then used in a high efficiency lithium acetate precipitation transformation method (see materials and methods) for transforming yeast strain #2 (*MED14-TAP*). The transformed yeast was plated on plates lacking uracil and incubated at 30°C for 2-4 days. The resulting colonies were then streaked to single colonies and tested for the presence of the HA tag on Rpb8 by Western blotting. The result is shown in Figure 23.



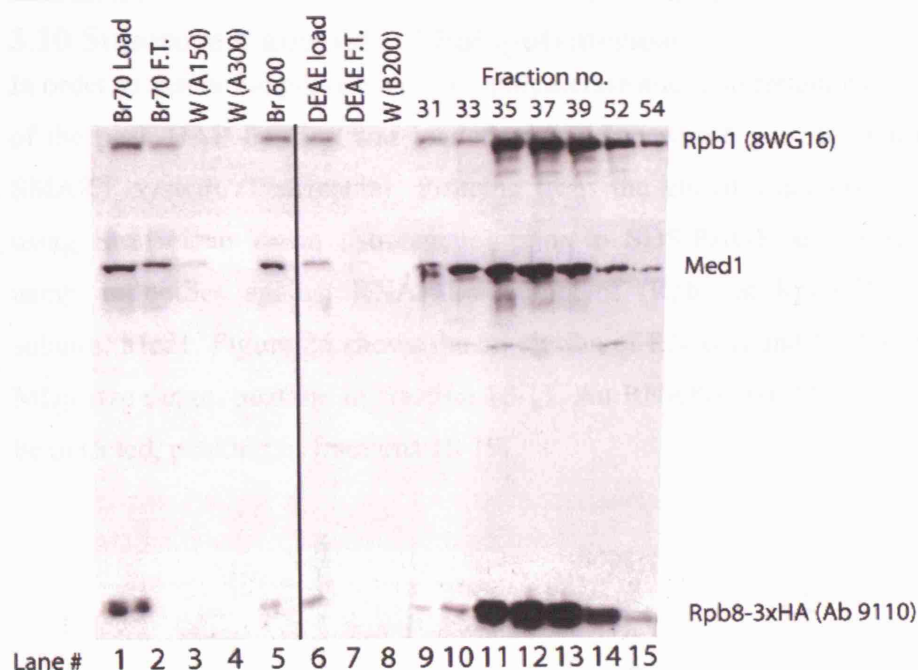
**Figure 23 Verification of the presence of the 3-HA tag on Rpb8**

Immunoblot of a 12% SDS-PAGE analysis of crude yeast extracts and a recombinant, purified HA-tagged version of Ctk1. The membrane was cut and blotted with anti HA (12CA5 lanes 1-3) or PAP against the TAP-tag on Med14 (lane 4-5). Ctk1-HA served as a positive control for the HA blot (lane 1). The negative control was W303 extract (lane 2 and 5) and extract made from the background Med14-TAP tag strain (lane 4). Only in the new Med14-TAP/Rpb8-3xHA is a band corresponding to the size of Rpb8-3HA visible (lane 3, lower band; the upper band is a non-specific cross-reacting band caused by capturing of the secondary antibody by the protein A tag on Med14-TAP)

### 3.9 Purification of Med14-TAP, Rpb8-3HA holopolymerase

After confirmation that Rpb8 and Med 14 were both tagged in the new *RPB8-3HA MED14-TAP* strain, HoloRNAPII was purified from it following the procedure outlined in Figure 11. An immunoblot of samples from the first two steps in the procedure (Biorex70 and DEAE Sephacel) is shown in Figure 24. Although some holoenzyme flowed through the BioRex-70 resin, the DEAE column in turn efficiently depleted Rpb1, Rpb8-3HA and Med1 proteins from the BioRex-70 elution pool (compare lanes 6 and 7). The co-elution of RNAPII and Mediator subunits in the batch elution step at 550 mM KOAc is shown in lanes 11 - 13. These fractions were pooled, and holoRNAPII further purified by chromatography on hydroxyapatite (HAP) column (Bio-Rad).

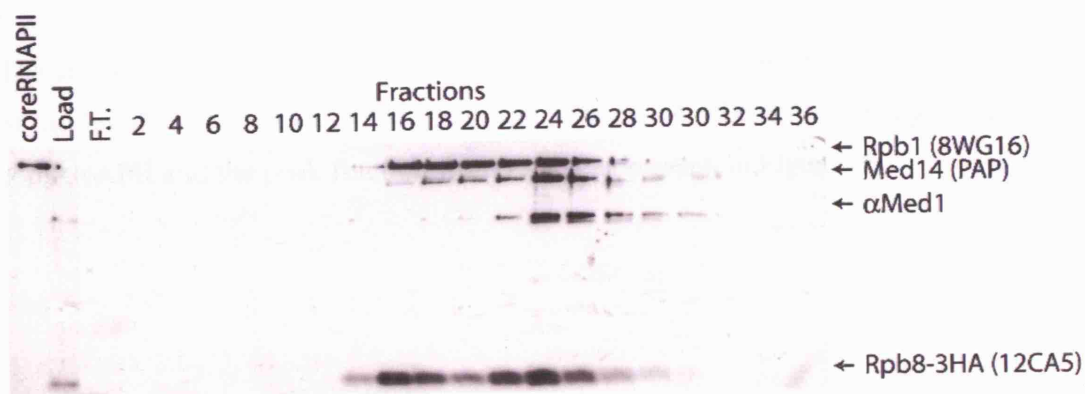




**Figure 24 Steps in the purification of holoRNAPII**

Immunoblot of 10% SDS-PAGE detecting RNAPII (Rpb1 & Rpb8-3HA) and Mediator (Med1). The DE550 peak of holoRNAPII emerged in fractions 35-39 (lane 11 - 13). Br70=BioRex-70 (Bio-Rad), DEAE=Diethylaminoethyl sepharose (Amersham), F.T.= unbound, W= Wash, A and B designates the buffer type and the subscript denotes the concentration of KOAc in mM. The line between lane 5 and 6 designates position where irrelevant lanes of the immunoblot have been removed for clarity.

An immunoblot across the subsequent hydroxyapatite elution profile indicated that the holopolymerase behaved similarly to the untagged version with coreRNAPII elution preceding holoRNAPII elution (see **Figure 25**). The separation of core and holoRNAPII is most easily seen by the Rpb8-3HA blot.

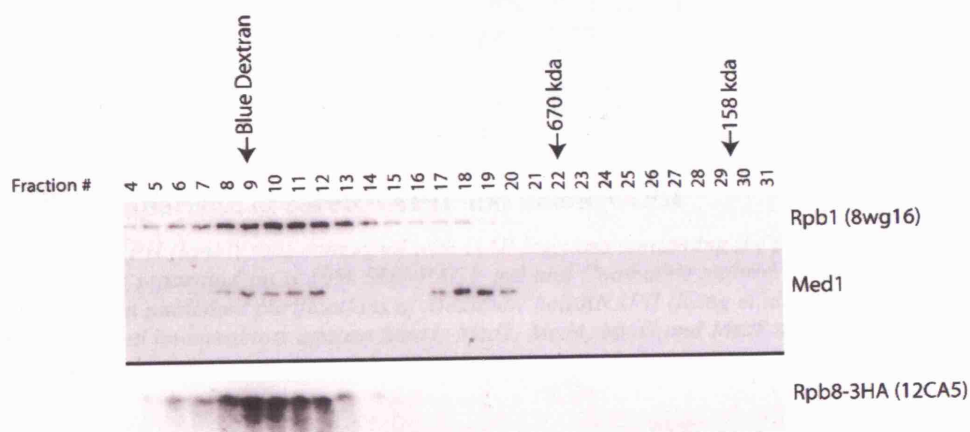


**Figure 25 Purification of holoRNAPII on Hydroxyapatite.**

The indicated HAP fractions were blotted to PVDF from 10% SDS-PAGE. Immunodetection using the indicated antibodies revealed the elution profile of coreRNAPII (Rpb8-3HA peak in fraction 16-18) and holoRNAPII (Rpb1, Med14, Med1 and Rpb8-3HA) peak in fraction 24).

### 3.10 Superose 6 analysis of holopolymerase

In order to verify the presence of holopolymerase and to ascertain its composition, some of the peak HAP fraction was loaded onto a Superose 6 gel filtration column using a SMART system (Pharmacia). Proteins from the eluted fractions were concentrated using Strataclean Resin (Stratagene) prior to SDS-PAGE and were immunoblotted using antibodies against RNAPII subunits of (Rpb1 & Rpb8-3HA) and Mediator subunit, Med1. Figure 26 shows the co-elution of RNAPII and Mediator subunits in the MDa size range, peaking in fraction 10-11. An RNAPII-free Mediator species can also be detected, peaking in fractions 18-19.



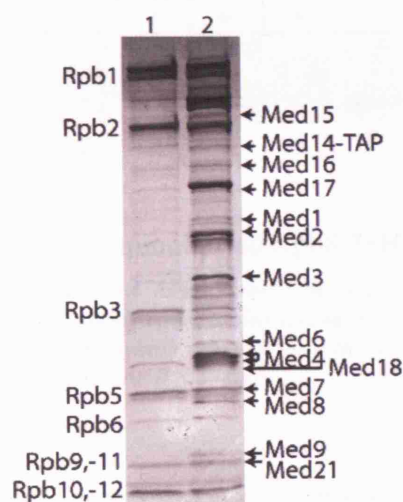
**Figure 26 Superose 6 analysis of holopolymerase**

*The purified holoRNAPII elutes from Superose 6 in the mega Dalton range in fractions 10-11. A peak of Mediator complexes devoid of RNAPII elute in fractions 18-19. Blue Dextran elutes at approximately 2 MDa. The line indicates position where the blot has been cut for clarity.*

The size of the purified holoRNAPII correlates well with literature which sets the size of the holoRNAPII to 1.9 MDa (Liu et al., 2001). A SDS-PAGE analysis comparing coreRNAPII and the peak fraction from HAP can be seen in Figure 27.

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The resulting holoenzymes were highly purified as indicated by Coomassie staining of fraction derived from the procedure (see Figure 27).



**Figure 27 Comparison of coreRNAPII and holoRNAPII**

Purified coreRNAPII (lane 1) was compared with HAP fractions containing the peak of holoRNAPII (lane 2). Proteins were separated on a 10% SDS-PAGE gel and Coomassie stained. Identities of bands have been inferred from published purifications of Mediator/ holoRNAPII (Kang et al., 2001; Kim et al., 1994; Li et al., 1995) and immunoblots against Med1, Med2, Med4, Med7 and Med8 as well as the TAP-tag on Med14 (data not shown).

Together, these results indicated that I now had purified RNAPII holoenzyme, tagged on Rpb8 and Med14, respectively.

### 3.11 Hyperphosphorylation of immobilised Rpb8-3xHA holoRNAPII

As mentioned above, optimisation of the buffer conditions allowed some of the 'old' Rpb2-HisHA holoRNAPII to be phosphorylated when immobilised to beads and incubated with ATP and TFIIH (Figure 28 lane 4). In contrast, immobilisations of the novel holoRNAPII complex via the triple HA tag on Rpb8 proved to enable TFIIH to quantitatively shift Rpb1 to the slower mobility band (Figure 28 compare lane 4 & 6).

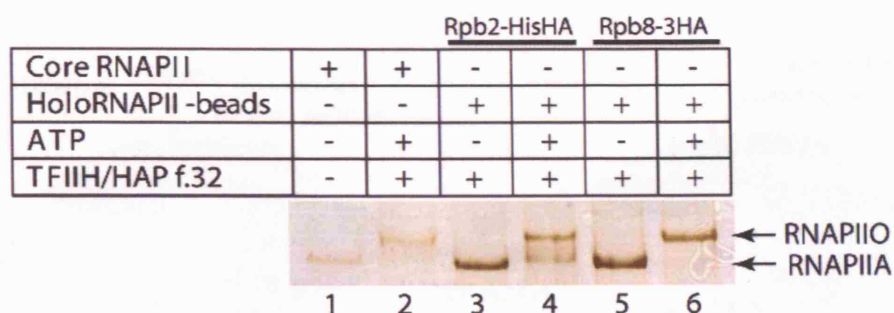


Figure 28 Hyperphosphorylation of immobilised Rpb8-3xHA-tagged RNAPII

Silver stained 5% SDS-PAGE analysis of kinase reaction. Hyperphosphorylation of holoRNAPII immobilised on 12CA5 affinity resin. Roughly equal molar amounts of holoRNAPII were used in a kinase assay and loaded on a 5% SDS-PAGE gel which was run until the 175 kDa marker reached the edge of the gel. The gel was silver stained to detect the Rpb1 band.

These results indicated that the Rpb8-3HA Med14-TAP holoenzyme could be used to study phosphorylation-dependent Mediator-RNAPII dissociation.

### 3.12 Phosphorylation by TFIH causes dissociation of Mediator and RNAPII

The new holoRNAPII carried a protein A tag, as part of the TAP tag on Med14. Protein A binds firmly to the constant region of most antibodies and therefore, in order to measure Mediator dissociation from immobilised RNAPII, it was necessary first to remove the Protein A part of the TAP-tag by TEV-protease treatment, to avoid immobilising holoRNAPII by the Med14-TAP tag on the 12CA5 resin.

To now test the hypothesis that hyperphosphorylation of the CTD leads to destabilisation of holoRNAPII, the affinity tag on Rpb8 was used to affinity purify and immobilize Med14(Rgr1)-TAP holoRNAPII on a 12CA5 (anti HA) antibody (Covance) directly cross-linked to Affigel-15 (Amersham). The modified procedure for dissociation assays is delineated in **Figure 29** and details can be found in Materials and Methods.



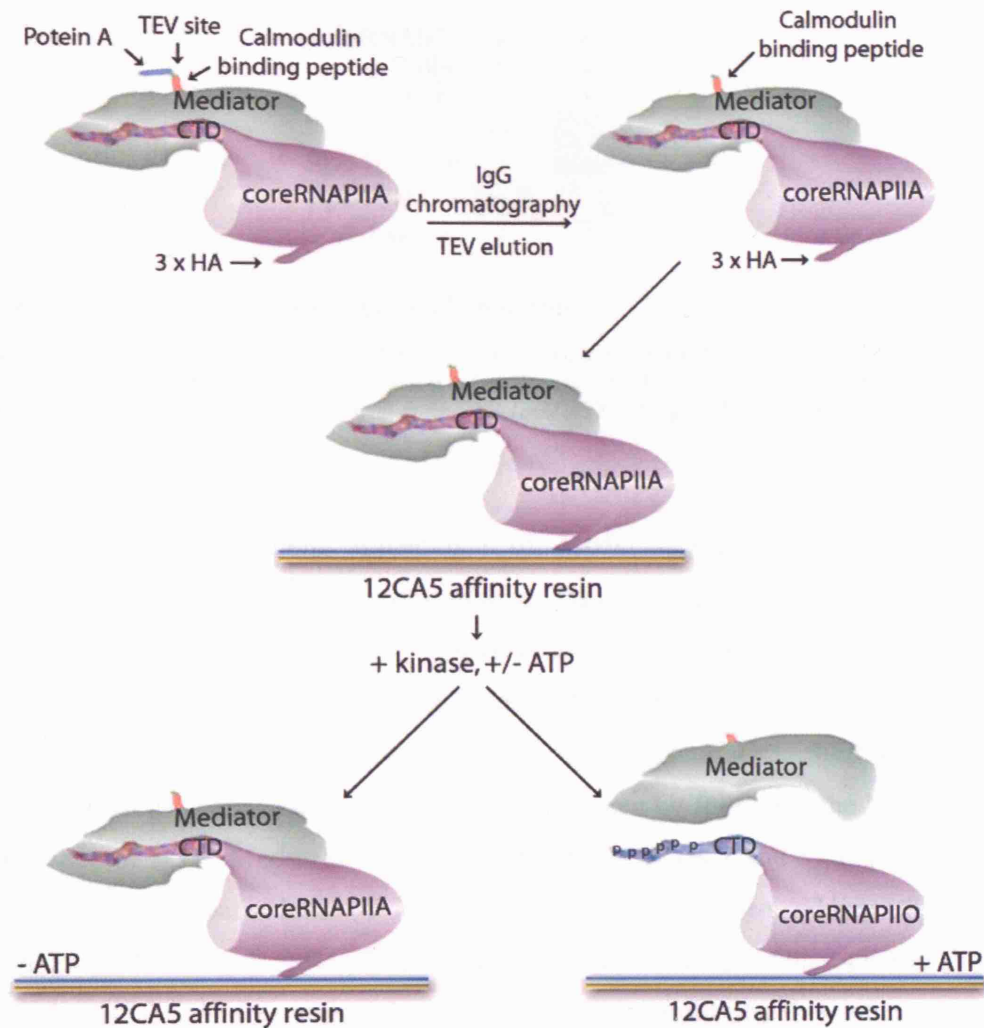
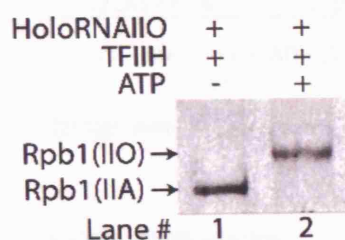


Figure 29 Dissociation assay using Rpb8-3HA/Med14-TAP holoRNAPII

*Dissociation assay using doubly tagged holoRNAPII and 12CA5 resin* The TEV protease treatment of holoRNAPII serves to remove the protein A part of the TAP tag which would otherwise bind Mediator to the 12CA5 beads.

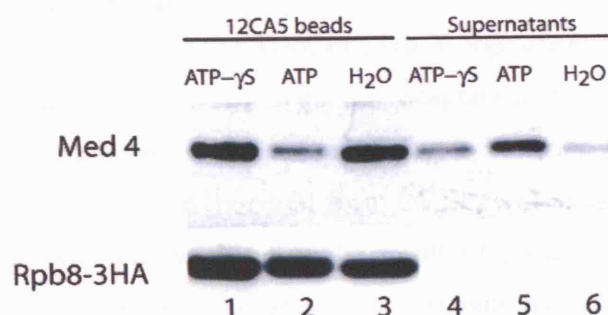
The complexes were immobilised and washed with 300 cv (3 ml) binding-buffer 1, and then washed into phosphorylation-buffer 1. TFIIH kinase with or without ATP was now added to hyperphosphorylate the RNAPII CTD. As a control for the effect of the kinase activity and correctness of the reagent mix, a parallel control reaction was always performed in the absence of ATP. Successful phosphorylation was indicated by the mobility shift of a parallel control reaction loaded on a 5% SDS-PAGE gel which was silver stained (see Figure 30).



**Figure 30 Hyperphosphorylation control reaction**

*Silver stained 5% SDS-PAGE analysis. For the dissociation assays, a parallel reaction was always run to make sure that hyperphosphorylation had indeed taken place. The ATP-dependent mobility shift in 5% SDS-PAGE is evident in lane 2. Reaction was done with 1mM ATP, 20 ng TFIIH, 100 ng holoRNAPII per reaction.*

The phosphorylation-dependent appearance of Mediator specific subunits in the supernatant was detected by immunoblotting of supernatants with Med4 polyclonal antibodies, while the presence of RNAPII on the resin was monitored by immunoblotting using 12CA5 antibodies against Rpb8-3xHA. The result of such a dissociation experiment is shown in Figure 31. Near quantitative, ATP dependent, dissociation of Med4 from immobilised RNAPII was seen by following the Med4 band: in the presence of ATP, Med4 leaves the resin (compare lanes 2 and 3) and appears in the supernatant (compare lanes 5 and 6). Interestingly, when adding the non-hydrolysable ATP analogue ATP- $\gamma$ S, a small, but detectable level of dissociation above background was observed (compare lane 4 and 6). This could possibly indicate some effect on holoRNAPII integrity of ATP binding per se. This possibility was investigated further by repeating the experiment with different nucleotides and the ATP analogue AMP-PnP, as well as different salt concentrations in the exchange buffer but unfortunately, no clear conclusion could be drawn from these preliminary experiments (not shown). More importantly, no significant holoRNAPII dissociation was visible in the presence of TFIIH but absence of ATP (compare lane 5 and 6), indicating that both ATP and TFIIH is needed, and that the presence of TFIIH per se does not cause holoRNAPII dissociation.



**Figure 31 Dissociation of Mediator from immobilised holopolymerase by phosphorylation with HAP fraction containing TFIIF**

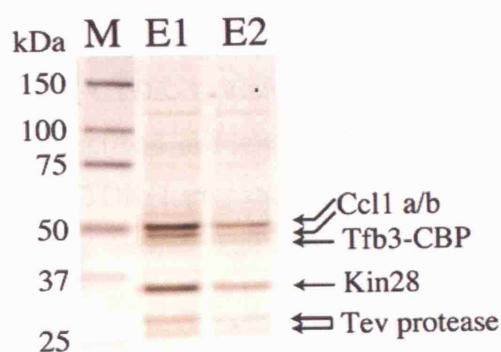
A dissociation assay analysed by 4-12 % SDS-PAGE and immunoblot using 12CA5 for the Rpb8-3xHA tag and anti-Med4 for the Mediator complex. HoloRNAPII was immobilised on Affigel-12CA5 beads and washed. In all reactions, 1  $\mu$ l holoTFIIF (HAP32) was added as well as either H<sub>2</sub>O, the non hydrolysable ATP analogue ATP- $\gamma$ S (1 mM), or ATP (1mM). Significant ATP-dependent dissociation of Med4 to the supernatant is seen only when adding ATP (lane 5).

The observed ATP dependent dissociation of holoRNAPII fits well with the observed change in phosphorylation status of the Rpb1 CTD at the stage of promoter clearance where RNAPII must dissociate from the promoter 'scaffold structure' (Yudkovsky et al., 2000) to transcribe the mRNA coding sequence. Primarily Ser5 is phosphorylated at this stage, with Ser2 only becoming phosphorylated later in the transcription cycle (Cho et al., 2001; Komarnitsky et al., 2000; Svejstrup, 2004). Although CTD phosphorylation appeared to be sufficient for dissociation in the above experiments, a possible (active) role in the dissociation of other subunits of TFIIF, such as the Rad3 and Ssl2 helicases, could not be ruled out. TFIIFK (Kin28/Ccl1/Tfb3) is the sub-module of TFIIF that harbours the CTD kinase Kin28, and this subunit interacts with Rpb1 and Rpb2 *in vitro* (Feaver et al., 1994; Takagi et al., 2003). In order to establish whether phosphorylation by TFIIFK is sufficient to elicit holoRNAPII dissociation, I therefore decided to purify the TFIIFK complex. Moreover, having observed that TFIIF phosphorylation leads to holoRNAPII dissociation, it became interesting to investigate whether dissociation requires phosphorylation at the residue specifically targeted by this kinase (Serine 5), or whether Serine 2 phosphorylation might also be able to dissociate holoRNAPII. The CTD kinase, CTDK-1 predominantly phosphorylates Ser2 *in vitro* (Jones et al., 2004) and *in vivo* (Cho et al., 2001). I therefore decided also to purify the CTDK-1 complex.



### 3.13 Purification of TFIID

A Tfb3-TAP strain obtained from the Kornberg lab (Borggrefe et al., 2001) was used as source material for purification, following an adaptation of the TAP tag protocol (Rigaut et al., 1999). The details of the procedure can be found in Materials & Methods. The TFIID complex was initially purified from 25 litres of yeast culture and a silver stain of a 4-12% SDS-PAGE analysis of the result of proteolytic elution from IgG-sepharose is shown in Figure 32. The gel shows two elution fractions (E1 and E2) and, as can be seen, the preparation is very pure, and suitable for use in the dissociation assay.



**Figure 32 Purification of TFIID**

A silver stain of 4-12% SDS-PAGE. TFIID was purified from a yeast strain expressing a TAP tagged Tfb3 protein using the procedure described in materials and methods. The Ccl1 subunit runs as a doublet probably caused by a site of alternative translation initiation at the beginning of the CCL1 ORF (Svejstrup et al., 1996a). Auto-proteolysis is the probable reason for TEV protease to appear as a doublet (Kapust et al., 2001).

### 3.14 Purification of CTD kinase 1

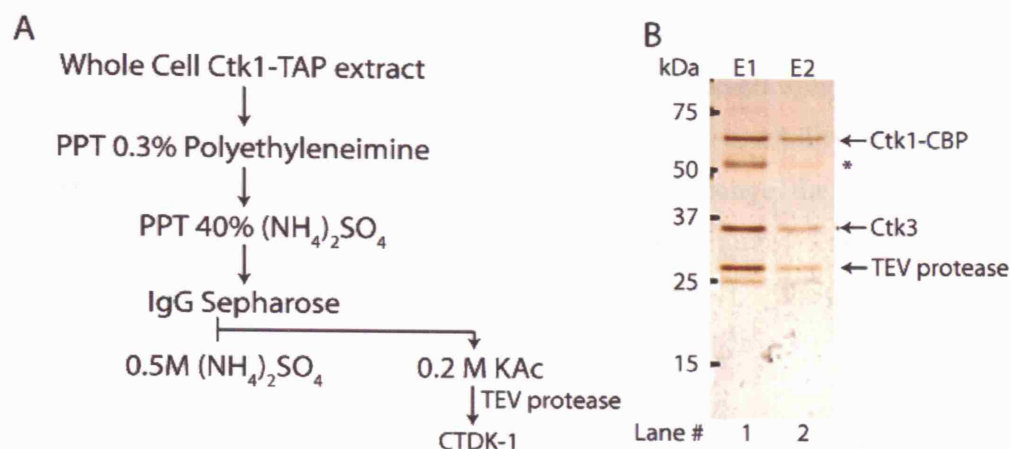
#### 3.14.1 CTDK-1 purification from CTK2-TAP yeast

A W303 strain expressing Ctk2-TAP was available from the lab freezer stock. This was used in attempts to purify active CTDK-1 kinase. The protocol followed was the generic TAP tag protocol described in Materials and Methods, and both IgG- and calmodulin-sepharose resin steps were employed in the purification. The elution fractions from the calmodulin column were tested using a kinase assay, but no activity was detected (not shown). The second largest CTDK-1 subunit Ctk2 is related to cyclin C in human (Sterner et al., 1995) and acts to regulate the kinase activity of the TFIID subunit Ctk1. CTDK-1 was originally reported to be a low abundance complex in yeast (Lee and

Greenleaf, 1989), but more recently Ctk2 was reported to occur abundantly in yeast nuclear extract as two separate forms; a phospho-protein, which is unstable (5 min half life), and a distinct unphosphorylated form, which is stable (Hautbergue and Goguel, 1999). Given that the affinity tag in the *Ctk2-TAP* strain was placed on the most labile subunit, and given that all attempts at purification from this source had failed, I decided to try purification using one of the other, more stable subunits as location for an affinity tag. I therefore finally used a Ctk1-TAP strain for purification of the CTDK-1 complex.

### ***3.14.2 Purification of CTDK-1 from Ctk1-TAP yeast***

A yeast strain (strain #1, see Materials & Methods) expressing a TAP tagged version of the Ctk1 protein was purchased (Cambio, Cambridge, UK) and used for the production of Ctk1-TAP protein. The CTDK-1 kinase complex was purified by the TAP purification procedure, as described in Materials and Methods, following the protocol outlined in Figure 33. The CTDK-1 preparation is very pure at this stage, though the complex does not seem to be entirely stoichiometric, as active CTDK-1 is a tri-mer consisting of Ctk1, Ctk2 and Ctk3 and only two subunits are clearly visible. Judged by the molecular weight markers, the lower subunit is Ctk3. The reason that the purified complex is sub-stoichiometric with regard to Ctk2 fits the reported fast degradation of Ctk2 upon phosphorylation (Hautbergue and Goguel, 2001). Nevertheless, and more important, this pure preparation was a very active CTD kinase (see Figure 34) implying that the Ctk2 subunit was indeed present.

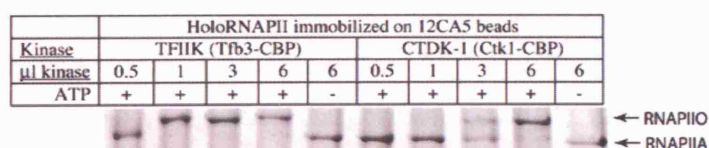


**Figure 33 Purification of CTDK-1 by Ctk1 affinity chromatography**

Silver stained 10% SDS-PAGE analysis of elution fractions (500  $\mu$ l fractions, 10  $\mu$ l loaded per lane) from IgG sepharose. Panel A outlines the purification procedure. Panel B shows two elution fractions from the IgG column. The band marked with an asterisk is probably due to insufficiently cross-linked IgG heavy chain dissociating from the beads.

### 3.15 Assay of CTD-kinase activity of CTDK-1 and TFIIK

The purified TFIIK and CTDK-1 complexes were now tested in a kinase assay to establish how much of each kinase was needed to quantitatively shift the Rpb1 band from holoRNAPIIA, immobilised on Affigel15-12CA5 beads. Kinase reactions using this immobilized holoRNAPII in the presence of 1 mM ATP (final concentration) were then performed.



**Figure 34 Activity titration of TFIIK and CTDK-1**

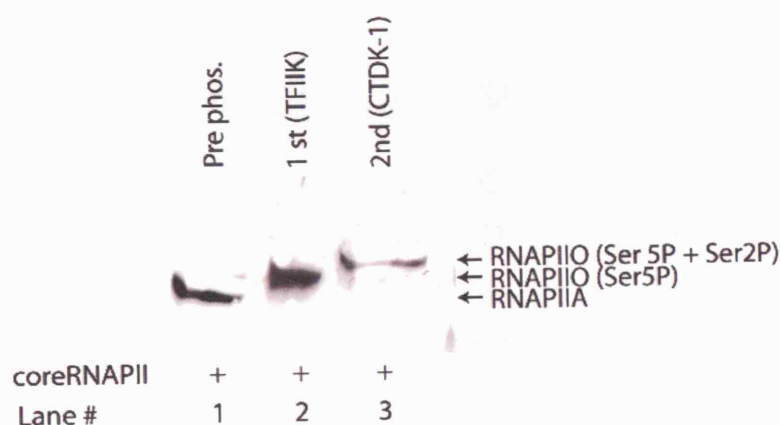
Silver stained 5% SDS-PAGE analysis of a kinase assay. HoloRNAPII was immobilised on 12CA5 beads before addition of kinases and reaction buffer as described in Materials and Methods. The concentration of TFIIK was 56 ng/ $\mu$ l and CTDK-1 was 67 ng/ $\mu$ l. ATP concentration was 0.5 mM. Both TFIIK and CTDK-1 is able to cause a shift in electrophoretic mobility of Rpb1.

### 3.16 Specificity of CTD kinases

A gel shift assay was performed in which phosphorylation of RNAPII using saturating amounts of TFIIK was used to shift the position of Rpb1 in 5% SDS-PAGE. Subsequent phosphorylation of RNAPIIO (Ser5P), using purified CTDK-1, caused a super-shift of RNAPIIO which implies that CTDK-1 targets additional positions other than Ser5 in the

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repeat sequence. In other experiments, Somesh Baggavalli hyperphosphorylated RNAPIIA to the IIO form by using saturating amounts of either the TFIIK or CTDK-1 kinase (purified as described in this thesis). Subsequent de-phosphorylation using the purified Ser5 specific phosphatase Ssu72, only elicited faster mobility (to the IIA level) of RNAPII phosphorylated by TFIIK, but failed to change the migration of the RNAPIIO band created by CTDK-1 phosphorylation (see Figure 35 and (Somesh et al., 2005). These results are consistent with Ser2 target specificity of the purified CTDK-1.



**Figure 35 Super-shift of RNAPII upon double phosphorylation**

Silver stain of 5% SDS-PAGE analysis. CoreRNAPIIA was phosphorylated by purified TFIIK in a kinase assay as described in Materials and Methods. RNAPIIO (Ser5P) was purified by spin column gel-filtration and an aliquot served as substrate for a CTDK-1 kinase reaction. Reactions done at 1 mM ATP, using 67 ng CTDK-1 and 50 ng TFIIK in a volume of 50  $\mu$ l.

Taken together, the data thus suggests that the preferential *in vitro* target specificity of the purified TFIIK and CTDK-1 kinases differ. As I now had purified different CTD kinases I went on to test i) whether dissociation could be achieved by the kinase complex (TFIIK) of TFIIH alone, and ii) if dissociation due to phosphorylation depended on the site of phosphorylation, using CTDK-1. A kinase assay was set up using TFIIK to phosphorylate the CTD of holoRNAPII immobilised on 12CA5-Protein A Sepharose. The result is shown in **Figure 36**. The first lane is the input beads without treatment. Although there is a higher background level of Mediator in the supernatant in this experiment in the absence of ATP (lane 3), upon ATP-dependent phosphorylation, Mediator specific subunits do accumulate in the supernatant (see Med4 blot, compare lane 3 and 5). The background may, in part, stem from contaminating, free mediator contaminating the TFIIH/HAP fraction, since this fraction was used as control for this



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particular experiment. However, lanes 6-9 show the same experiment, this time adding the highly purified TFIIF. A clear, ATP-dependent accumulation of Mediator subunits is observed in the supernatant (compare lane 7 and 9). This indicates that the activity of the TFIIF kinase complex is sufficient to dissociate holoRNAPII in an ATP dependent manner, and strongly suggests that the other ATPases (helicases) in TFIIF do not play an indispensable role for the dissociation.

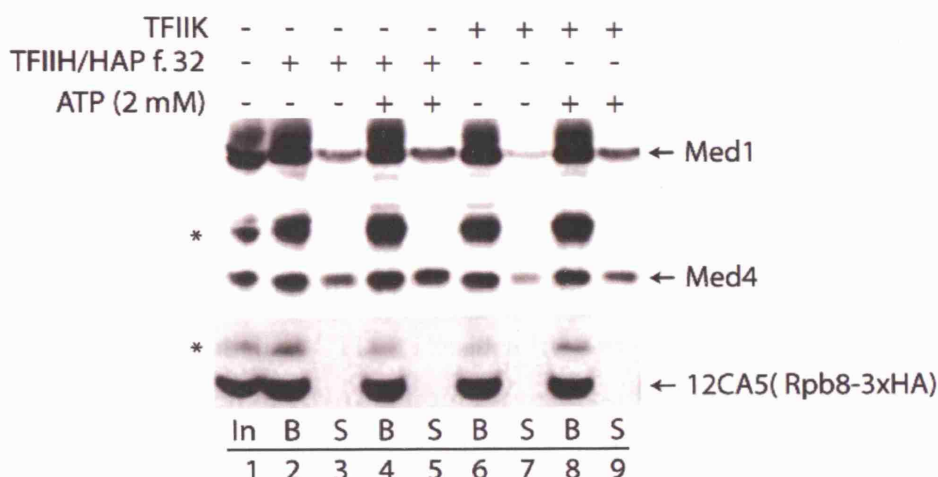


Figure 36 Phosphorylation by TFIIF or TFIIF causes holoRNAPII dissociation

An immunoblot of a dissociation assay. 12CA5-ProteinA-sepharose beads were used to immobilise holoRNAPII. Where indicated, 60 ng of TFIIF or 150 ng TFIIF, and 2mM ATP was used to phosphorylate the CTD. In= Input beads, B= beads, S= supernatant. The bands marked with asterisks are non-specific bands, probably arising from the IgG heavy and light chains of insufficiently cross-linked 12CA5.

The dissociation experiment was repeated, this time by immobilising holoRNAPII on IgG beads by utilising the TAP tag on Med14 (Rgr1). The detection of coreRNAPII dissociating from the holoRNAPII complex was attempted by immunoblotting for Rpb8-3HA using the 12CA5 antibody. Unfortunately, the signal in these first experiments was too weak for reliable detection. This could possibly be due to the peak holoRNAPII fraction from HAP containing a significant amount of free Mediator devoid from RNAPII. This idea is supported by the data of Figure 26. Super stoichiometric Mediator complex in the HAP fraction could potentially provide an excess of binding surfaces for RNAPII, so that even after its phosphorylation, it might remain associated with the beads.

Attempts to enrich for holoRNAPII was done by TEV protease treatment of holoRNAPII to remove the Protein A part of the TAP tag prior to capturing RNAPII-



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containing complexes on 12CA5 via the tag on Rpb8. Subsequently, holoRNAPII (now free from free Mediator complex) was eluted with HA peptide. Finally, holoRNAPII was recaptured on calmodulin resin utilising the remaining part of the TAP tag on Mediator, and the dissociation assay was performed again. Unfortunately, no signal from dissociation of RNAPII was observed. The remaining concentration of the remaining holoRNAPII was possibly too low after the many purification steps. The possibility that RNAPII has some non-specific association with the resin which precludes its dissociation can also not be ruled out at this point. In order to save precious protein for other, more important experiments, this experiment was not repeated with more protein.

The problem in the above experiment seemed to be detection of the dissociating RNAPII by immunoblotting for one of its subunits, so one possibility was to detect directly the dissociating RNAPII by performing the assay with labelled ATP, following the behaviour of the labelled CTD. The idea here was that if phosphorylation of the CTD caused dissociation of RNAPII from the matrix-associated Mediator complex, the labelled polymerase would primarily be detected in the supernatant. The result of this experiment is shown in Figure 37.

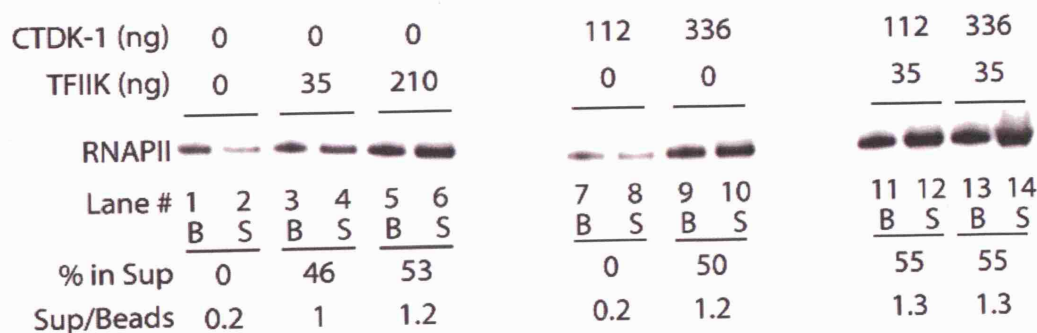


Figure 37 Dissociation of RNAPII from immobilised Mediator

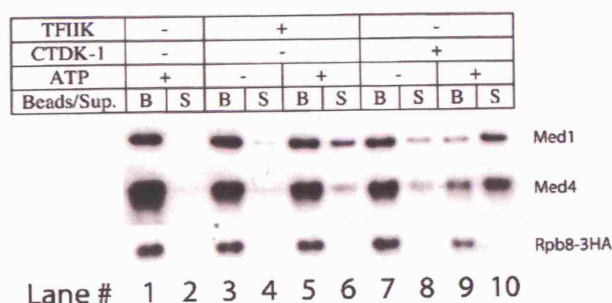
*Autoradiogram of 10% SDS-PAGE analysis of a dissociation assay. Please note that at this percentage, SDS-PAGE gels do not resolve the IIA from the IIO band. HoloRNAPII was immobilised by the Med14-TAP tag on IgG-resin. Amounts and identity of kinases in each reaction as designated. Assay was done with 200  $\mu$ M cold ATP and 0.5  $\mu$ Ci  $\gamma$ - $^{32}$ PATP/ $\mu$ l and the autoradiogram was quantified by densitometry using the ImageJ (<http://rsb.info.nih.gov/ij/>) program. The background level of dissociation (lane 2) was subtracted from other supernatant values before calculating how much of the total radioactivity in each assay was recovered in the supernatant.*

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Increasing amounts of kinase leads to increasing dissociation of RNAPII to the supernatant (compare lane 4 & 5 for TFIIK and lane 8 and 10 for CTDK-1), indicating that phosphorylation indeed leads to holoRNAPII dissociation. When the CTD kinases are combined (lane 11-14), even more phosphorylated RNAPII is recovered in the supernatant (lane 12 & 14) possibly indicating some degree of synergy between Ser2 and Ser5 phosphorylation. The result supports the previously reached conclusion that dissociation of holoRNAPII can be caused by CTD hyperphosphorylation of both Ser2 and Ser5.

### 3.17 Urea exacerbates destabilisation of holoRNAPII

Previous work by others showed that holopolymerase immobilised on anti-Rgr1-Sepharose can be separated into RNAPII and Mediator by extensive washing with buffer containing 1M urea. In contrast, the Mediator complex itself sustains its integrity in up to 2 M Urea (Kang et al., 2001). In an attempt to exacerbate the destabilising effects of CTD phosphorylation, the dissociation assay was done using buffers containing 300 mM Urea. The result is shown in Figure 38.



**Figure 38 The dissociation of Mediator from RNAPII is exacerbated by 0.3 M urea**

*Both TFIIK and CTDK-1 can cause dissociation of Mediator from immobilised holoRNAPII. 60ng of TFIIK and 70 ng of CTDK-1 were used in each reaction to phosphorylate approximately 100ng of holoRNAPII*

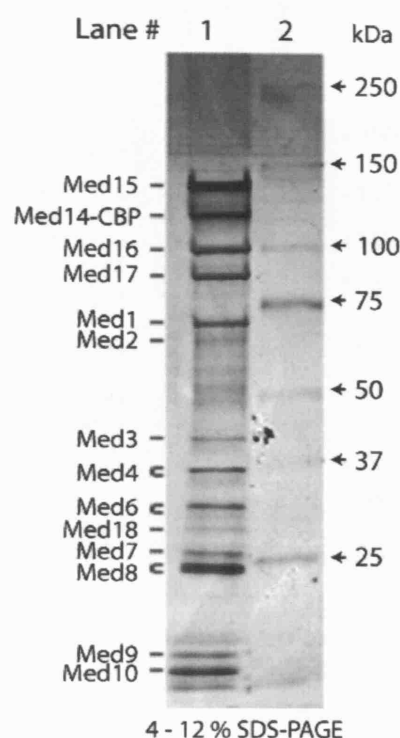
Inclusion of 0.3 M urea does not destabilise the holoRNAPII complex on its own (Figure 38, lane 2). However, in combination with TFIIK and ATP, significant dissociation of Mediator is observed (lane 6). The effect is even clearer when using purified CTDK-1. In this case, near quantitative dissociation of Mediator from the 12CA5 beads is observed (lane 10). Taken together, the results described so far indicate that CTD phosphorylation of not only Ser5, but also Ser2, results in dissociation of the Mediator-RNAPII complex.

### **3.18 CTD phosphorylation and CTD binding proteins**

Throughout the transcription cycle, changing CTD phosphorylation patterns serve to orchestrate the timely interaction of CTD interacting proteins involved in diverse aspects of transcription. However, CTD interacting proteins can regulate phosphorylation as well. For example, the presence of Mediator stimulates the CTD kinase activity of TFIIF around 40 fold, ostensibly due to a conformational effect on the CTD making it a better substrate for the kinase (Kim et al., 1994). HoloTFIIF has been reported to interact *in vitro* with the Mediator subunit Med15 (Gal11) (Sakurai and Fukasawa, 2000) and hence, an allosteric effect relayed through holoTFIIF to attenuate Kin28 kinase activity, could play a role in stimulation as well. The precise molecular mechanism of the stimulation of TFIIF kinase by Mediator remains unknown. In order to look closer at the mechanism of stimulation of CTD phosphorylation I decided to purify the Mediator complex and see if it was able to stimulate the TFIIF complex that I had purified.

### **3.19 Purification of Mediator**

To study potential functional interaction between the various CTD kinases purified and the Mediator complex, Mediator was purified from Med14 (Rgr1)-TAP yeast following a protocol developed by Ralph Davis (personal communication) described in Materials and Methods. The end result is shown in Figure 39.

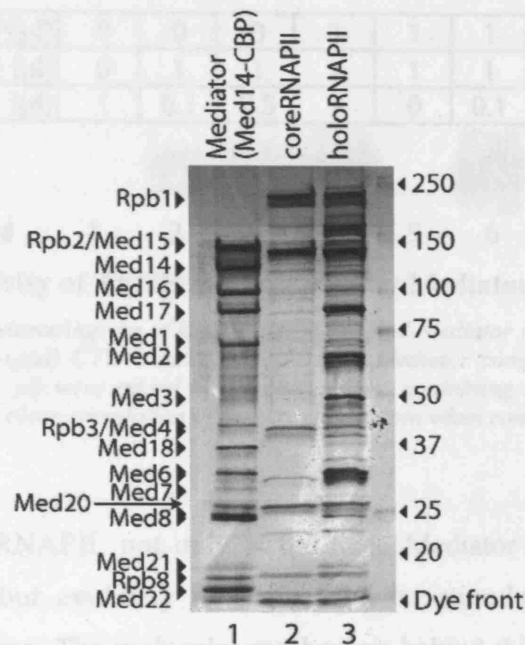


**Figure 39 Purification of the Mediator complex**

*Coomassie stained 4-12% SDS-PAGE gel. Mediator complex (Rgr1-CBP) was purified using a Med14-TAP strain of yeast as described in materials and methods. 10 $\mu$ l/lane of the pooled elution fractions from IgG was loaded on the gel.*

### 3.20 Mediator, coreRNAPII and holoRNAPII preps compared

To compare the purified complexes, these were separated on a 4-12% SDS-PAGE gel and Coomassie stained. The result is shown in Figure 40. Bands arising from RNAPII and Mediator are visible in the holoRNAPII peak fraction. Other bands are also apparent, indicating that the holoRNAPII peak is not completely pure at this point. However, please note that the dissociation protocol invariably included one or two further affinity purification steps, after the point showed in Figure 40, so that the dissociation experiments were performed with even purer proteins.



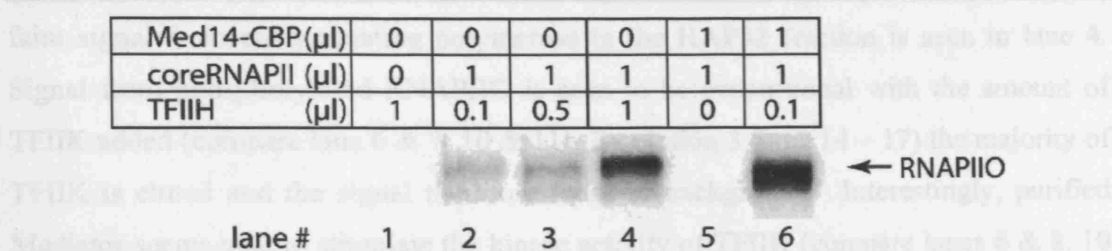
**Figure 40 Comparison of purified protein complexes**

A Coomassie stained 4-12% SDS-PAGE analysis of purified Mediator, coreRNAPII and holoRNAPII eluted from 12CA5 beads (see above). Approximately equal molar amounts were loaded in each lane. To detect proteins the gel was Coomassie stained. The identity of bands have been assigned based on published apparent Mw (Myers et al., 1998; Takagi et al., 2005) as well as on immunoblots for Rpb1, Rpb3, Rpb8, Med14, Med1, Med2, Med4, Med7 and Med8.

### 3.21 Kinase activity of TFIIF is stimulated by Mediator

To test whether the purified Mediator was able to stimulate the activity of holoTFIIF, a kinase assay was set up. An autoradiogram showing the result is shown in Figure 41. CTD phosphorylation increases in proportion to added holoTFIIF, indicating that these amounts of TFIIF fall within a linear range (see lanes 2 to 4). Mediator on its own has no detectable CTD kinase activity (see lane 5) and as expected from the literature (Kim et al., 1994; Myers et al., 1998), CTD phosphorylation by TFIIF kinase is greatly stimulated by the presence of purified Mediator complex (compare lane 2 and 6).

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**Figure 41 Kinase activity of TFIIH is stimulated by Mediator**

5% SDS-PAGE analysis autoradiogram of kinase assay. Purified Mediator (60 ng/ $\mu$ l), coreRNAPII (300 ng/ $\mu$ l) and holoTFIIH (20ng/ $\mu$ l) CTD kinase activity by the Mediator complex. Increasing amounts of holoTFIIH (0.1, 0.5 and 1  $\mu$ l) were added in kinase reactions containing 300 ng coreRNAPII with or without 60 ng Mediator. A clear stimulation of activity can be seen when comparing lane 2 and 6.

Thus, when binding RNAPII, not only is the huge Mediator complex able to avoid occluding the CTD, but evidently it is also able to stimulate the phosphorylation reaction of TFIIH kinase. The molecular mechanism behind this is unknown. A priori, the association of Mediator with coreRNAPII might "present" the CTD to potential interacting proteins and, in so doing, cause it to be a better substrate for CTD kinases.

### 3.22 Mediator stimulates TFIIK activity

Surprisingly, the stimulation by Mediator of TFIIH kinase activity was also a feature inherent to the TFIIK sub-module. The elution fraction from the IgG column was tested using a kinase assay where purified Mediator was added (see Figure 42).



**Figure 42 Stimulation of Kin28 kinase activity by Mediator**

Kinase assay. Autoradiogram of 5% SDS-PAGE analysis of batch elutions from TFIIK purification. The CTD kinase was purified from extract from Tfb3-TAP expressing cells. The profile of batch elutions from IgG was tested by kinase assay (see materials and methods) using labelled [ $\gamma^{32}$ ]PATP and stimulation of kinase activity by adding purified Mediator complex. Mediator strongly stimulates CTD phosphorylation by TFIIK (compare lane 10 and 12). Mediator concentration was 60 ng/ $\mu$ l.

In this particular experiment, the highly active hydroxyapatite fraction 32 (TFIIH/HAP f. 32) was used as a positive control with purified coreRNAPII as substrate (lane 1). A



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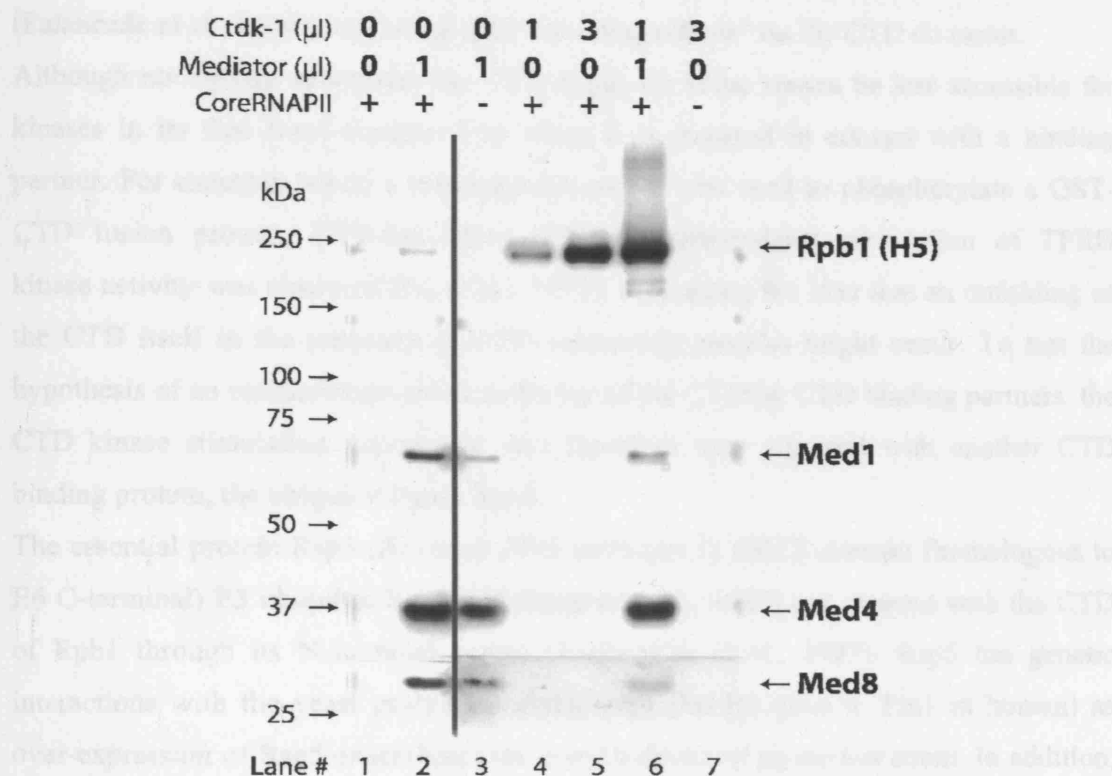
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faint signal from contaminating polymerase in the HAP32 fraction is seen in lane 4. Signal from phosphorylated RNAPIIO is seen to be proportional with the amount of TFIIK added (compare lane 6 & 7, 10 & 11). By elution 3 (lane 14 – 17) the majority of TFIIK is eluted and the signal therefore fades to background. Interestingly, purified Mediator seems able to stimulate the kinase activity of TFIIK (compare lanes 6 & 8, 10 & 12). Previously, stimulation by TFIIH has been observed (Kim et al., 1994) but the stimulation by the TFIIK sub-complex outside of the context of TFIIH had not at this point been demonstrated. The stimulation of Kin28 kinase activity by purified Mediator is interesting because physical contact *in vitro* between TFIIH and Mediator has only been reported between Tfb1 and amino acids 866–929 of the Mediator tail subunit Gal11 (Sakurai and Fukasawa, 1998), and in the context of TFIIK there is no Tfb1 present to mediate any possible allosteric regulation of Kin28 kinase activity. Although interaction between TFIIK subunits and Mediator may have gone undetected, the basis for the stimulation could also lie in a modification of the CTD substrate due to binding of Mediator to RNAPII. Such binding may cause the CTD to unfold as previous reports in the human system have suggested (Lu et al., 1992).

### 3.23 CTDK-1 kinase is stimulated by the presence of Mediator

The previous experiment suggested that under the conditions of the experiment, binding of Mediator to the CTD serves to expose the CTD to interaction with TFIIK. Such an unfolding mechanism might serve to enable the access of other kinases as well. In an attempt to see whether purified Mediator could stimulate CTDK-1 activity as well, a kinase assay was set up using coreRNAPII, CTDK-1 (Ctk1-CBP) and Mediator (Med14-CBP), and the reaction products were analysed by immunoblotting using antibodies against Mediator (Med1, Med4 and Med8) as well as against the Ser2 phosphorylated Rpb1 (H5 antibody). The result is shown in Figure 43. Lane 1 shows that the substrate was unphosphorylated while lane two demonstrates that the purified Mediator is devoid of Ser2-specific kinase activity. (lane2). Increasing amounts of Ctdk-1 results in increasing phosphorylation (lane 4 & 5), indicating that under these conditions, the reaction is not yet saturated. Surprisingly, adding purified Mediator to the lowest amount of Ctdk-1 kinase, stimulates kinase activity significantly (compare lane 4 and 6). The H5-crossreactive signal indicates that the phosphorylation is specific for Ser2, as expected for Ctdk-1, and gives rise to band shift in the top of the gel (lane 6). Stimulation of CTDK-1 activity by Mediator has not previously been reported.

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**Figure 43 CTD kinase 1 is stimulated by purified Mediator**

Immunoblot of 4-12% SDS-PAGE analysis of kinase assay. The grey line between lane 2 & 3 denotes position where image was cut to remove lanes for clarity. The kinase assay was performed as described in materials and methods. CTD kinase 67 ng/μl, RNAPII 300 ng/μl, 1, Mediator 40 ng/μl, 1 mM ATP Med 1, Med 4 and Med8 are Mediator subunits. The H5 antibody recognises Ser2 phosphorylation as well as Ser5 phosphorylation. The membrane was stripped and re-probed with H14 antibody recognising Ser5 phosphorylation and but no signal was detected (not shown). The results are consistent with specificity of CTDK-1 for Ser2 phosphorylation.

The ability of Mediator to stimulate the kinase activity of both TFIIF and CTDK-1 prompted experiments to investigate the basis for the stimulation. In theory, allosteric interaction between Mediator subunits and TFIIF and CTDK-1 could be stimulating their kinase activity, but no such interactions have been reported in literature.

As described in the introduction, a host of proteins, (GTF's, isomerase's, capping machinery, splicing factor) are known to interact with the CTD, in the transition from initiation to elongation phase of transcription. Similarly, the large Mediator complex must bind to the CTD at the promoter, creating a notion of a very "busy" CTD domain. It is currently unknown how many CTD binding proteins associate with the CTD at any one time, but the activity of Fcp1 has been reported to be inhibited *in vitro* by steric



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hindrance from Pin1 binding to the CTD rather than by Pin1's isomerisation activity (Palancade et al., 2004), indicating that "crowding effects" on the CTD do occur.

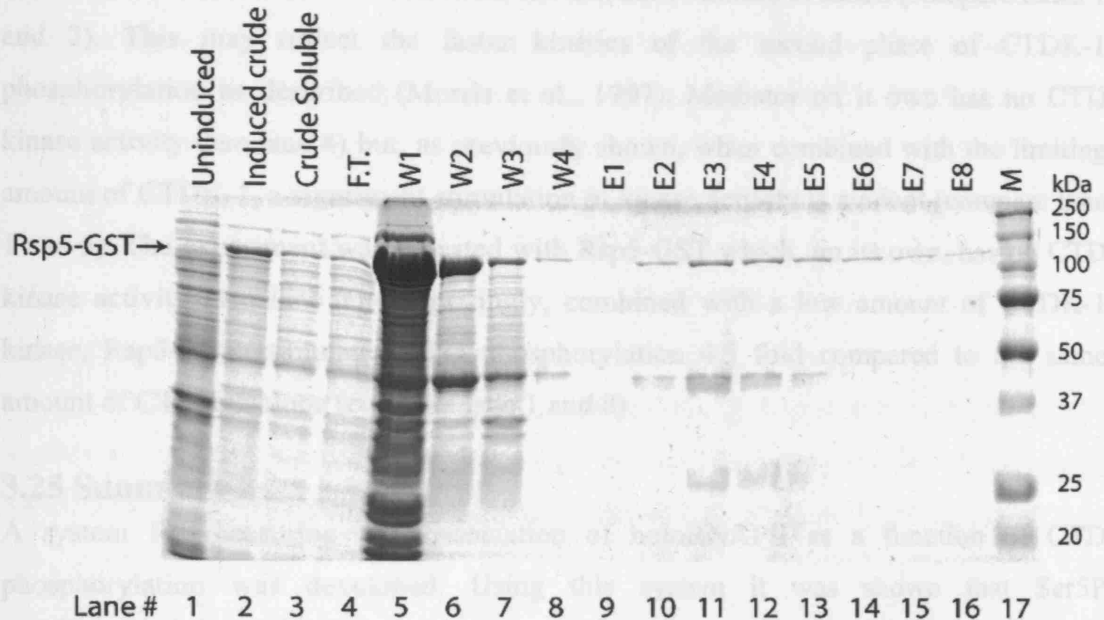
Although not rigidly structured, the CTD might for some reason be less accessible for kinases in its free form compared to when it is engaged in contact with a binding partner. For example, when a transcription system was used to phosphorylate a GST-CTD fusion protein, GTF-dependent (RNAPII-independent) stimulation of TFIIF kinase activity was observed (Lu et al., 1992), supporting the idea that an unfolding of the CTD itself in the presence of CTD contacting proteins might occur. To test the hypothesis of an enhancement of accessibility of the CTD by CTD binding partners, the CTD kinase stimulation experiment was therefore now repeated with another CTD binding protein, the ubiquitin ligase Rsp5.

The essential protein Rsp5 (Reverses *SPt3* mutation 5) HECT-domain (homologous to E6 C-terminal) E3 ubiquitin ligase (Huibregtse et al., 1995) can interact with the CTD of Rpb1 through its N-terminal region (Huibregtse et al., 1997). Rsp5 has genetic interactions with the yeast prolyl-isomerase Ess1 (homologous to Pin1 in human) as over-expression of Rsp5 exacerbates the growth defect of an *ess1-ts* strain. In addition, Ess1 and Rsp5 were reported to be competing for the same binding site on Rpb1. The data suggested that Rsp5 and Ess1 have opposing effects on transcription. (Wu et al., 2001)

Studies *in vitro* showed that Rsp5 interaction with RNAPII leads to the ubiquitylation of lysine 695 of Rpb1 (Somesh et al., 2005). RNAPII in an elongation complex is a much better substrate for ubiquitylation than free RNAPII. Furthermore, the reaction is strictly dependent on the phosphorylation status of the CTD inasmuch as Ser5 phosphorylation is completely inhibitory under all conditions while Ser2 phosphorylation is tolerated, both in the free- and the ternary-complex form of RNAPII (Somesh et al., 2005). Rsp5 has been reported to be associated with the TFIID complex purified from *S. cerevisiae* (Auty et al., 2004). The phosphorylation-dependent RNAPII-specific activity of Rsp5, as well as its association with a complex that interacts with RNAPII before its hyperphosphorylation and promoter clearance suggested that Rsp5 might be one candidate for stimulation of kinase activity. Hence, a plasmid expressing a GST-Rsp5 fusion protein was procured from the Huibregtse lab and Rps5 was purified as described in Materials and Methods. The result is shown in Figure 44. Induction of Rsp5-GST is efficient (compare lane 1 and 2), but part of the protein is in the insoluble fraction as indicated by the preferential loss of the Rsp5 band by centrifugation of the crude extract

## Results Part I

(compare lane 2 and 3). Purification from the soluble fraction still gives rise to a peak of Rsp5-GST at the expected molecular weight (see lanes 11 -12).



**Figure 44 Rsp5-GST purification.**

Coomassie stained 4-12% SDS-PAGE. F.T.= Unbound, W=washes each concentrated from 2 ml using Strataclean resin, E=elution, M= marker. Full-length Rsp5-GST peaks in E3 and runs approximately at the expected size for the fusion protein. A peak of possible break-down products between 37 and 50 kDa co-elutes with the Rsp5-GST peak.

### 3.24 Mediator & Rsp5 stimulate CTDK-1 kinase activity

To investigate whether Rsp5 can stimulate CTD kinase activity like Mediator, a kinase assay was set up using Rsp5 and Mediator to stimulate the phosphorylation of the CTD by Ctk1 kinase. The result of this assay is shown in Figure 45.

Rsp5-GST (μl)	-	-	-	-	-	1	1	1
Med14-CBP (μl)	-	-	-	1	1	-	-	-
CoreRNAPII (μl)	1	1	1	1	1	-	1	1
CTDK-1 (μl)	1	2	4	-	1	-	-	1
Fold phosph.	1	7.6	10.5	0	3.1	0	0	4.5

**Figure 45 Mediator and Rsp5 stimulate CTDK-1 activity**  
An autoradiogram of a 5% SDS-PAGE analysis. Rsp5-GST and Mediator stimulates the kinase activity of CTDK-1. Concentrations of proteins are: CTDK-1 67 ng/μl, RNAPII 300 ng/μl, Mediator (Med14-CBP) is 40 ng/μl, Rsp5-GST is 200 ng/μl.

Phosphorylation of the CTD is increased as more CTDK-1 added (see lanes 1-3). The signal is more than doubled when twice the amount of kinase is added (compare lanes 1 and 2). This may reflect the faster kinetics of the second phase of CTDK-1 phosphorylation as described (Morris et al., 1997). Mediator on its own has no CTD kinase activity (see lane 4) but, as previously shown, when combined with the limiting amount of CTDK-1, a significant stimulation of kinase activity is evident (compare lane 1 and 5). The experiment was repeated with Rsp5-GST which, on its own, has no CTD kinase activity (see lane 7). Surprisingly, combined with a low amount of CTDK-1 kinase, Rsp5-GST stimulates CTD phosphorylation 4.5 fold compared to the same amount of CTDK-1 alone (compare lane 1 and 8).

### 3.25 Summary

A system for measuring the dissociation of holoRNAPII as a function of CTD phosphorylation was developed. Using this system it was shown that Ser5P phosphorylation through the action of holoTFIIH is able to dissociate Mediator from immobilised RNAPII. Kinases with different specificity were purified and it was found that both Ser2P and Ser5P are able to dissociate holoRNAPII, whether it is immobilised via Rpb8-3HA (RNAPII) or Mediator14-TAP subunits. In the course of the work, a stimulatory effect of Mediator on both TFIIF and CTDK-1 activity was uncovered. Further, in preliminary experiments, the ubiquitin ligase Rsp5 was shown to stimulate CTD phosphorylation by CTDK-1. The continued project will characterise these mechanisms in more detail, and, for example, investigate whether Rsp5-GST can stimulate TFIIF activity. The results also prompted me to investigate whether conformational changes in the CTD domain might occur as a result of Mediator and/or Rsp5 binding (see below).

## Part II

The second part of the thesis describes attempts to measure in real time, the effects of CTD phosphorylation and CTD-binding proteins on the structure of holoRNAPII complex and the CTD itself. The focus in the text below is on the development of assays to address these questions and on solving technical issues that have arisen during this initial work. This part of the thesis should be considered “work in progress”.

### **4 HoloRNAPII dissociation monitored using Biacore**

The interaction between Mediator and polymerase is dynamic since mediator is able to 'jump' between immobilised polymerases (Svejstrup et al., 1997). Moreover, although Mediator normally also interacts with other domains of RNAPII, the CTD interaction is sufficient as the free Mediator complex is also able to bind a GST-CTD fusion protein immobilised on beads (Myers et al., 1998). As Mediator has been found to change conformation to a more elongated form upon interaction with RNAPII (but not with the CTD peptide alone) (Asturias et al., 1999) the kinetics of binding presumably are different in each case. Hyperphosphorylation of the CTD causes dissociation of the holopolymerase complex, but the mechanism of dissociation is not clear.

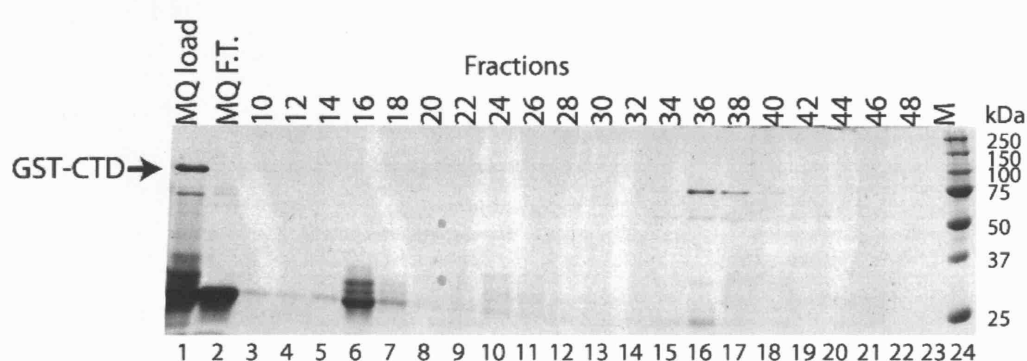
The mechanism of dissociation might be caused purely by a reduction in the affinity of Mediator for the hyperphosphorylated CTD, which should translate into an increased dissociation constant upon hyperphosphorylation. If the mechanism involves a conformational change of Mediator or polymerase upon hyperphosphorylation, one might expect the association constant to decrease upon hyperphosphorylation of RNAPII. Biacore measures in real time the changes in the total mass of molecules close to the surface of a Biacore "chip", using a physical phenomenon called surface plasmon resonance. A "chip" is a small capillary flow device which is divided into 2 or 4 individual flow-cells. These can be subjected to individual treatment and can be measured on simultaneously. The specific level of response (or response units) in a Biacore experiment is proportional to the mass of the molecules applied. The surface of the CM5 chip is covered by a layer of dextran which can be activated chemically to covalently capture ligand proteins that are expected to interact with candidate analytes which are dissolved in buffer that continually flows over the chip. The four phases of a Biacore experiment normally consists of a capture phase where the surface is covered in ligand and a baseline signal is acquired. This is followed by an association phase where analyte is passed over the chip while the rate of accumulation on the surface is measured. A dissociation phase where the concentration of analyte in the buffer is dropped to zero and the rate of dissociation of material from the surface is measured, and finally a regeneration phase where the surface is purged from bound ligand and the baseline is re-acquired. The real time measurement of on-rate (rate of accumulation) and off-rate (loss of protein from the ligand covered surface) makes possible the estimation of binding kinetics for the interaction of interest. To remove noise, all measurements are normalized to a reference cell (on the same chip), which is covered in a suitable

## Results Part II

negative control ligand that does not interact with the analyte. The analysis of Mediator interaction with activators has been done previously (Lee et al., 1999) but those experiments did not address the RNAPII-Mediator interaction. To study the mechanism of dissociation in greater detail by Biacore experiments I needed to purify a number of proteins. The experiments planned include using RNAPII-3xHA as a ligand and purified Mediator as the analyte. In addition, to study the interaction of Mediator with the CTD-domain only, purification of a GST-CTD fusion protein was required.

### 4.1 Purification of GST-CTD

In order to test whether the Mediator purified would interact with a GST-CTD protein and to investigate the effect of CTD phosphorylation on the interaction, it was necessary to purify GST-CTD. A plasmid (pGEX-4-T1, Amersham Biosciences) expressing glutathione-S-transferase fused to the yeast CTD sequence encompassing 26 repeats was obtained from the Proudfoot lab (described in (Barilla et al., 2001)). The purification of GST-CTD protein is described in materials and methods. After the GST step, some contaminating bands still persisted, making an additional purification step necessary. The protein eluted from the GST column was pooled and dialysed against buffer B<sub>50</sub> and loaded onto a MonoQ HR 5/5 strong anion exchange column (Amersham) which was subsequently developed with a gradient from 50mM KCl to 1M KCl as described in Materials and Methods. The result is shown in Figure 46.



**Figure 46 Purification of GST-CTD**

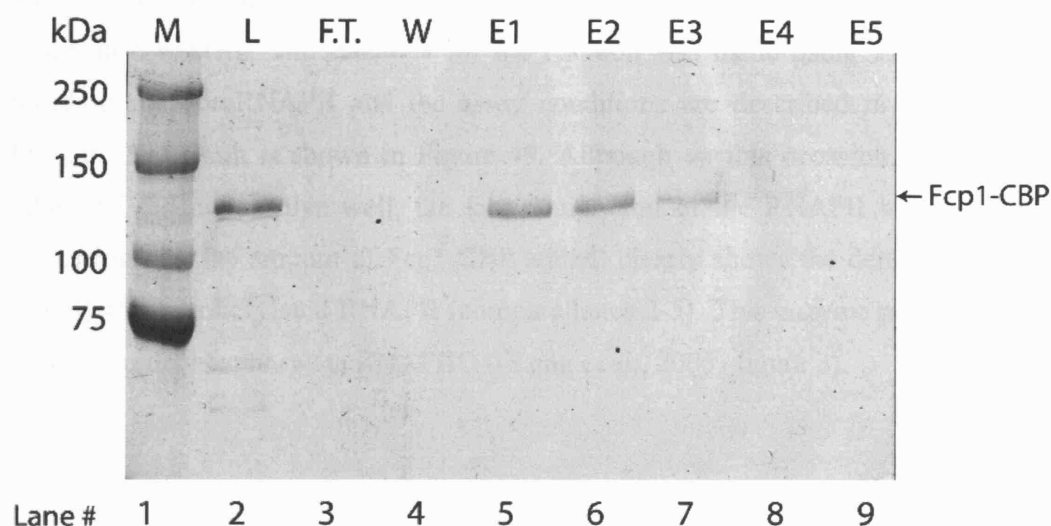
*Coomassie stained 10% SDS-PAGE analysis of GST purification. MonoQ purification of the pooled elution fractions from GST-resin. A fairly pure, full length GST-CTD fusion protein elutes in fractions 36-38 (see lane 16-17)*

## Results Part II

The contaminating band either flows through the MonoQ HR 5/5 column (lane 2) or elutes early in the salt gradient (lanes 5-7) resulting in the successful removal of the majority of contaminating bands from GST-CTD preparation. GST-CTD elutes in fractions 36-38.

### 4.2 Purification of FCP1

The CTD may adopt some specific conformation due to adaptive fit to interacting proteins. Two CTD specific phosphatases (Fcp1 and Ssu72) are associated with the transcription complex, and while Fcp1 *in vivo* has specificity for Ser2P (Cho et al., 2001), Ssu72 targets Ser5P (Krishnamurthy et al., 2004). Fcp1 has also been reported to have a non-CTD binding site in RNAPII (Chambers et al., 1995; Suh et al., 2005). To study if conformational dynamics of the CTD is affected by these activities, Fcp1 protein was purified from a yeast strain expressing a C-terminally TAP tagged version of the *FCPI* gene. The procedure followed was as described in materials and methods for TAP tag purification. A Coomassie stained a 7.5% SDS-PAGE analysis of the purification is shown in Figure 47. The elution fractions from the Calmodulin contain only one band, indicating that the preparation is very pure. s

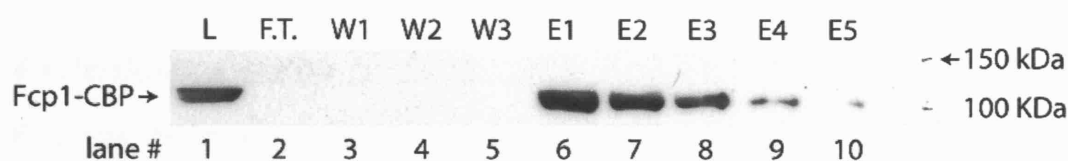


**Figure 47 Calmodulin step of Fcp1 purification**

A Coomassie stained 7.5% SDS-PAGE analysis of 10  $\mu$ l samples is shown. Fcp1-CBP protein eluted from IgG beads using recombinant TEV protease (lane 2) was loaded onto calmodulin resin (Stratagene), washed and eluted in batch (lane 5 – 9) as described in Materials and Methods. Analysis shows a single Coomassie stainable band indicating highly pure Fcp1-CBP.

## Results Part II

The theoretical molecular weight of Fcp1 based on the primary sequence is 83.4 kDa and the band purified runs between 100 kDa and 150 kDa. A purified recombinant hexa-histidine tagged version of the *S. cerevisiae* Fcp1 was observed to migrate at a molecular weight above 100 kDa (Kobor et al., 1999), and the CBP tag adds 7 kDa to the molecular weight of Fcp1-CBP. An immunoblot using a rabbit polyclonal antibody against Fcp1 confirmed the identity of the purified protein (see Figure 48)



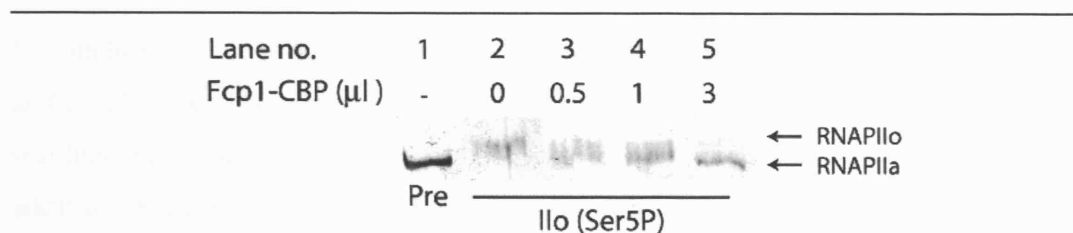
**Figure 48 Immunoblot analysis of the purification of Fcp1-CBP.**

The calmodulin-sepharose purification, batch washing and elution of Fcp1-CBP was analysed by 10% SDS-PAGE. The gel was immunoblotted and probed using a polyclonal rabbit antibody against FCP1. L= load on calmodulin resin, F.T. unbound fraction. W= wash fraction. E= elution fraction.

### 4.3 Test of FCP1 CTD phosphatase activity

The protein purified from an Fcp1-TAP tag expressing strain was tested for CTD phosphatase activity. The substrate for the reaction was made using TFIIFK to hyperphosphorylate coreRNAPII and the assay conditions are described in Materials and Methods. The result is shown in Figure 49. Although on this occasion, the 5% SDS-PAGE gel did not resolve well, the faster migration of the RNAPII band in the gel (proportional to the amount of Fcp1-CBP added) clearly shows the dephosphorylation of the Ser5 phosphorylated RNAPII (compare lanes 2-5). This enzyme preparation was also used to dephosphorylate RNAPIIO ((Kong et al., 2005) figure 3).

## Results Part II



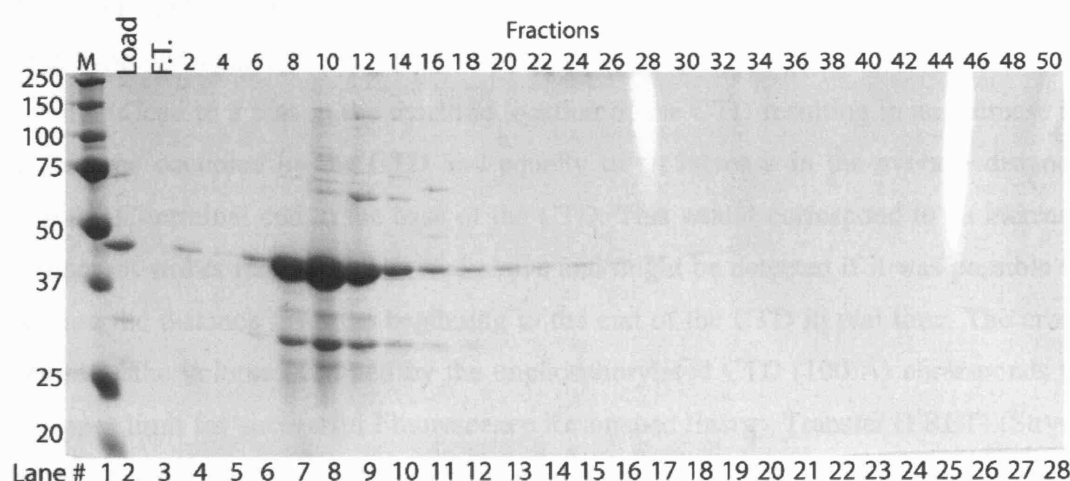
**Figure 49 Fcp1 activity assay.**

Silver stained 5% SDS-PAGE. Dephosphorylation by Fcp1-CBP of RNAPII phosphorylated at Ser5 using TFIIH as described in materials and methods (lanes 2 -5). Hypo-phosphorylated coreRNAPII is shown in lane 1. Amounts of Fcp1-CBP as indicated.

### 4.4 Purification of Ess1

Ess1 was shown by genetics to stimulate transcription elongation by isomerisation of the CTD repeats (Wu et al., 2003) and in addition to have a positive role in transcription initiation (Wilcox et al., 2004). Ess1 interacts *in vitro* with the phosphorylated CTD irrespective of the position of the phosphoserine in the individual repeats (Myers et al., 2001), and genetic interactions have been demonstrated between Ess1 and both Kin28 and CTDK-1 (Wilcox et al., 2004).

On this basis, it was interesting to test whether purified Ess1 could influence the dissociation of the holopolymerase complex after phosphorylation, and whether it could affect the structure of the CTD (see below). Ess1 was purified by GST-Sepharose affinity chromatography (see Materials and Methods) and the pooled elution fractions were applied to a MonoQ HR 5/5 column (Amersham) and developed with a gradient from 0.05 M KCl to 1 M KCl. The MonoQ elution profile is shown in **Figure 50**.



**Figure 50 MonoQ elution profile of GST-Ess1.**

Coomassie stained 10% SDS-PAGE. Elution fractions from the GST-sepharose step (see gel in Materials and Methods) were pooled, diluted and applied to a MonoQ FR 5/5 column. The procedure is described in details in Materials and Methods.



In conclusion, the reagents needed to study whether a change in holoRNAPII integrity and/or CTD conformation can be brought about by modification of CTD amino acid residues by phosphorylation, dephosphorylation or isomerisation are now at hand. In addition, possible effects on CTD conformation of binding of CTD interacting proteins (like Rsp5) can also be investigated.

### **4.5 Measuring CTD conformational change by FRET.**

As mentioned in the introduction, the issue of CTD conformation in relation to phosphorylation has been intensively studied by NMR analysis (Cagas and Corden, 1995; Harding, 1992; Jones et al., 2004; Kumaki et al., 2001), circular dichroism (CD) analysis (Bienkiewicz et al., 2000; Nishi et al., 1995) as well as by gel filtration and analytical ultracentrifugation (Zhang and Corden, 1991b). Furthermore, CTD conformation, attained by induced fit, have been suggested based on the crystal structures of CTD repeat peptides bound to CTD interacting domains (CID) from CTD phosphatases (Kamenski et al., 2004; Noble et al., 2005).

According to electron density measurements, the unphosphorylated RNAPII CTD occupies an area which is around 100 Å wide and has a volume roughly 4 times the calculated theoretical volume according to sequence, suggesting a dynamically changing CTD conformation (Meinhart and Cramer, 2004; Meredith et al., 1996). One prediction would be that in individual Rpb1 molecules at any given point in time the extreme C-terminal of the hypophosphorylated CTD (as well as the beginning of the CTD sequence) is located within this 100 Å wide volume.

If the hypothesis that specific Ser5 phosphorylation causes the CTD to stretch is correct, this might lead to a bias in the dynamic location of the CTD resulting in an increase in the volume occupied by the CTD and equally to an increase in the average distance from the C-terminal end to the base of the CTD. This would correspond to an increase in apparent stokes radius as described above and might be detected if it was possible to measure the distance from the beginning to the end of the CTD in real time. The cross section of the volume occupied by the unphosphorylated CTD (100 Å) corresponds to the upper limit for successful Fluorescence Resonance Energy Transfer (FRET) (Stryer and Haugland, 1967), and measurements of changes in average conformation of frayed DNA ends of DNA oligos has been done using fluorescence resonance energy transfer (FRET) (Danell and Parks, 2003). Though this was in a gas phase system, using FRET

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between chemical fluorophores it illustrates the potential use of the technique in the analysis of dynamic CTD conformations.

To measure CTD conformational change by FRET, the basic idea is to place fluorescent tags at each end of the CTD sequence and measure the change in FRET during and after hyperphosphorylation by TFIIH, TFIK or CTDK-1. Compaction or expansion of the CTD domain induced by binding of CTD interacting proteins may also be detectable by FRET. The measurement could take place either in the form of intensity based measurement of the decrease in fluorescence from the acceptor molecule as the donor-acceptor distance widens, or, alternatively, in the form of fluorescence lifetime imaging (FLIM) where a decrease in the excited-state lifetime of the donor is proportional with proximity of the acceptor fluorophore. . In FRET applications described in the literature, variants of the enhanced green fluorescent protein (EGFP) have often been used and especially the enhanced cyan fluorescent protein (ECFP) and the enhanced yellow fluorescent protein (EYFP) have been employed as a FRET pair. This pair has a Forster distance (distance where 50% of the emitted energy is absorbed by the acceptor) of approximately 49Å in water (Patterson et al., 2000). This value is calculated assuming a random orientation of the acceptor and donor fluorophores, a condition which is only approximated by the flexibility of the linker peptide and the CTD domain. A potential complication is the size of the EGFP proteins which is approximately 26.8 kDa (Genbank accession no. U55762, region 679..1398). Presumably, the larger the fluorophore, the greater its potential influence on the conformation of the CTD will be. Further, many of the GFP variants exhibit a propensity to multimerise increasing the probability of experimental artefacts (Tsien, 1998). An alternative approach is to use a chemical, the fluorescein-based biarsenical hairpin binder (FlAsH) (Griffin et al., 2000). FlAsH is a non-fluorescent derivative of the fluorochrome fluorescein which is inherently quenched in its free state, by two freely rotating arsenic groups. These groups can specifically and covalently bind a tetra cysteine motif [CCPGCC] in a recombinant protein of interest. This binding reaction, in turn, fixes the position of the arsenic groups which stops them from quenching fluorescein fluorescence, thus rendering the covalently attached group highly fluorescent (Griffin et al., 2000). The FlAsH reagent is commercially available from Invitrogen as Lumio green (excitation maximum 508nm, emission maximum 528nm cat. # LC6090). Moreover, a derivative of the red fluorescing fluorophore Resourfin (ReAsH), working by the same principle as FlAsH, is commercially available from Invitrogen as Lumio Red (excitation maximum 593 nm,

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emission maximum 608 nm cat. #12589-040). The active fluorophore molecule in both cases is small (500 Da) and diffuses readily into living cells with a marginal effect on survival (Adams et al., 2002; Griffin et al., 2000).

Based on the hypothesis of the compact CTD spiral (Meinhart et al., 2005) as well as the phosphorylation-dependent shift in apparent stokes-radius of a GST-CTD fusion protein, the CTD ought not need the rest of the RNAPII complex to display compaction and extension upon phosphorylation/dephosphorylation. On the other hand, CTD interacting proteins like Fcp1 and Rsp5 have also been reported to interact with non-CTD parts of the RNAPII complex. Rsp5 has been found in genome wide co-precipitation studies to interact with Rpb3, Rpb5 and Rpb6 (Ho et al., 2002), and Fcp1 was reported to have a binding site in Rpb4 (Kamenski et al., 2004; Kimura et al., 2002). Furthermore, the association of Fcp1 with the CTD was reported to be very transient (Suh et al., 2005) and thus for Fcp1 to stay associated with RNAPII *in vitro* the resulting influence (if any) of Fcp1 on CTD conformation may depend on Fcp1 contacting its binding site at the base of the Rpb4/Rpb7 sub-complex.

In order to investigate CTD conformational change both in the presence and absence of the rest of RNAPII the design of the FRET system to measure CTD conformational change followed two different, parallel approaches.

Firstly, to study the CTD compaction/expansion in isolation it was necessary to move the heptapeptide repeat sequence out of RNAPII context and I decided to fuse the CTD to GST for purification purposes. The second approach sought to place labels at either end of the Rpb1 CTD sequence to be able to gauge what effect the RNAPII complex would have on CTD conformation and indeed interaction with binding partners. In addition, this construct would have the advantage of potential *in vivo* use for RNAPII detection by fluorescence microscopy.

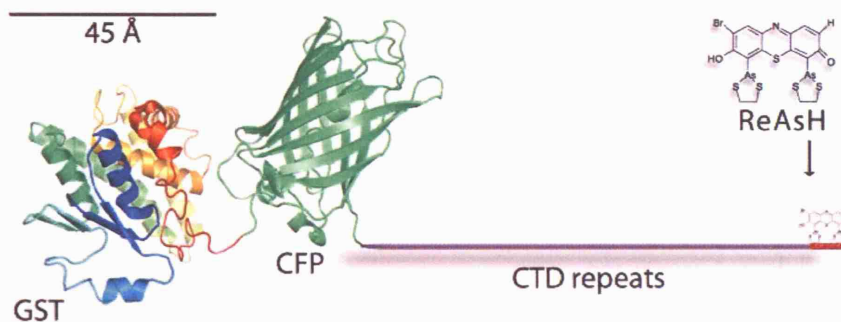
### ***4.6 A CFP-based model to study CTD conformation by FRET***

During development of the tetra-cysteine tag, it was initially thought that an alpha helical context (Ac-WEAAAREACCRECCARA-NH<sub>2</sub>) would favour the coordination of cysteine with the FLaSH reagent (Griffin et al., 2000). Further work later established that the two helix breaking amino acids Pro and G resulted in the most efficient labelling when they were put in the middle of the tetra cysteine motif (Adams

et al., 2002). Furthermore, shortening of the peptide to Ac-WDCCPGCCK-NH<sub>2</sub> had negligible effect on labelling efficiency. The Lumio kit vectors (Invitrogen) encode a tetra cysteine tag with flanking triple-glycine motifs, suggesting that the tag works well in a region of high flexibility. For use in this system, I decided to use the C-terminal tetra-cysteine sequence GGCCPGCCGGG-Stop.

FRET between CFP and FAsH *in vitro* has been reported previously (Adams et al., 2002), and by tetra-cysteine tagging of the  $\beta$ -tubulin subunit Tub2 in yeast, its *in vivo* labelling (using the FAsH reagent) was demonstrated through real-time imaging of living cells (Andresen et al., 2004). FRET has been used to monitor agonist-induced conformational change of the adenosine A2(A) G-coupled receptor in HeLa cells *in vivo* (Hoffmann et al., 2005). In that instance, the C-terminal of the receptor protein was fused to cyan fluorescent protein (CFP) and a tetra-cysteine tag was inserted into a loop in the structure and labelled by adding the fluorescein derivative FAsH to the culture medium. This suggests that FRET can be used to study intra-molecular distances in proteins.

Partly based on this information, a new design was created where the CTD sequence was tagged on either end with CFP or the Lumio tag, respectively. The N-terminal of this construct was in turn to be fused to GST for easy purification, and the Gateway system (Invitrogen) was employed for the construction. A drawing of the construct is shown in Figure 51 and details on the construction can be found in Materials and Methods. That the construct had the proper sequence was verified by sequencing before expression and purification (as described in Materials and Methods).

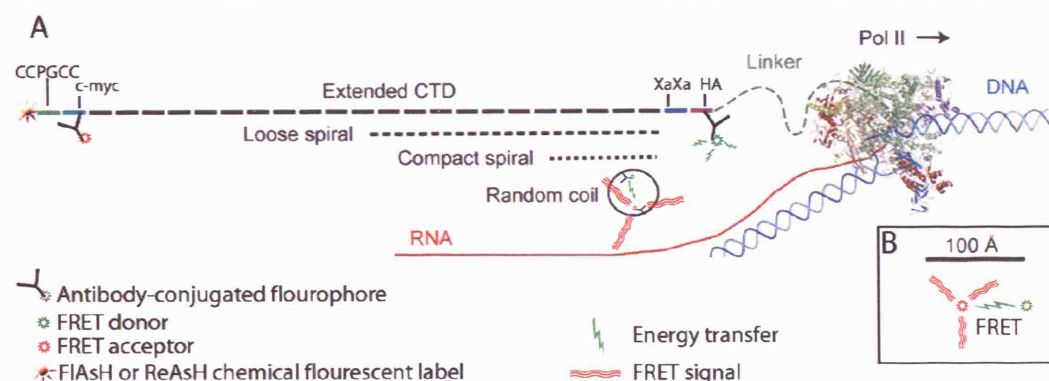


**Figure 51 Illustration of the GST-CFP-CTD-CCPGCC construct**

*Principle of the FRET assay. The drawing illustrates the position of CFP and the tetracysteine tag at either end of the CTD. Here, the labelling reagent is illustrated as ReAsH which labels tetra-cysteine tags exactly like FAsH but has a red-shifted spectrum compared to FAsH. The ReAsH is a reagent derived from the fluorochrome Resorufin, which can also be used to label the CCPGCC tag. The GST and CFP molecules are rendered from PDB structures 1UA5 and 1OXD, respectively, and are drawn to the illustrated scale. The CTD and ReAsH are not drawn to scale. The CTD would be 6.5 times the illustrated length, if drawn to scale.*

#### 4.7 Construction of RNAPII carrying a doubly tagged CTD

A strain of yeast (strain #6 see Materials and methods) was created which survives on a plasmid expressing a HA tagged version of Rpb1 (Li and Kornberg, 1994). Among other things, RNAPII isolated from this strain was previously used to localise the beginning of the CTD repeat sequence in RNAPII (Meredith et al., 1996), I used this strain as the basis for the construction of the FRET system in the context of normal RNAPII. The HA tag in this strain is placed between the CTD repeats and the linker sequence that connects the CTD to the main body of the RNAPII complex. Also present is a double factor Xa cleavage site C-terminally to the HA epitope. This site enables the use of a protease for control experiments to ensure that FRET measured between closely spaced CTD ends is indeed based on intra-molecular interaction and not purely by crowding effects *in vitro*. The location of the beginning of the repeat sequence can thus be labelled using a fluorescently labelled anti-HA antibody (12CA5). The extreme C-terminal end of the CTD can be tagged with the tetra-cysteine CCPGCC sequence to enable it to be labelled using the FAsH chemical. I also incorporated an additional c-myc epitope next to the CCPGCC tag to enable the alternative use of fluorescently labelled c-myc antibody (9E10) in the system.



**Figure 52** Illustration of FRET assay

RNAPII and the CTD illustrations are drawn to the scale shown in panel B. Panel A: Cartoon shows hypothetical CTD conformational classes and their difference in theoretical CTD length (Meinhart et al., 2005). Also shown are the positions of the CCPGCC-tag and the epitope tags, engineered onto the ends of the CTD, for the FRET based assay. Figure B illustrates that FRET between donor and acceptor is most effective at inter-fluorophore distances of less than 100Å. In the extended conformation or even in the loose spiral confirmation, the ends of the CTD would hence be too far apart to support efficient FRET. On the other hand, if the CTD adopts a random coil or a compact spiral structure (for example (after phosphorylation or upon binding of a protein partner), a FRET signal from end to end is possible. Figure A is adapted from (Meinhart et al., 2005). HA & c-myc =epitope tags, XaXa double factor Xa protease site.

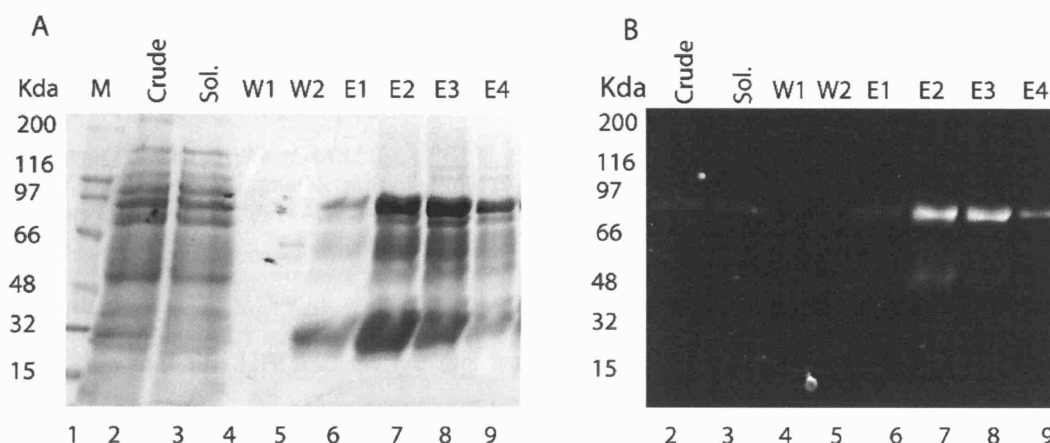
The working principle of the system for the detection of apparent CTD length by FRET is illustrated in Figure 52. The construction of the doubly tagged Rpb1 is described in Materials and Methods. Briefly, a PCR product encoding the myc/tetra-cysteine tag and a URA marker was used to transform strain #3 by electroporation as described in Materials and Methods. Recombination served to replace the C-terminal sequence of the plasmid-borne RNAPII with the combined c-myc/tetra-cysteine tag.

#### 4.8 Purification of GST-CFP-CTD-CCPGCC

The GST-CFP-CTD-CCPGCC protein was purified as described in Materials and Methods. The result is shown in Figure 53. The samples were stained with the Lumio green reagent as described in Materials and Methods before SDS-PAGE analysis. The gel was filmed in a Las 3000 gel documentation system (panel B) before being Coomassie stained (panel A). This purification of GST-CFP-CTD-CCPGCC appeared to result in a significant amount of contaminating bands, perhaps due to the loading exceeding the binding capacity of the GST affinity column and subsequent insufficient washing of the column. Alternatively, the bands represent C-terminal degradation of the CTD domain (and hence the CCPGCC tag) as in-gel labelling with the Lumio green

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chemical reveal that the majority of the smaller bands are untagged (compare A and B). Whatever the cause of the contamination, panel B clearly demonstrates that the tetra-cysteine motif in GST-CFP-CTD-CCPGCC can be labelled in-gel using the Lumio kit from Invitrogen.



**Figure 53 Labelling of GST-CFP-CTD-CCPGCC in-gel**

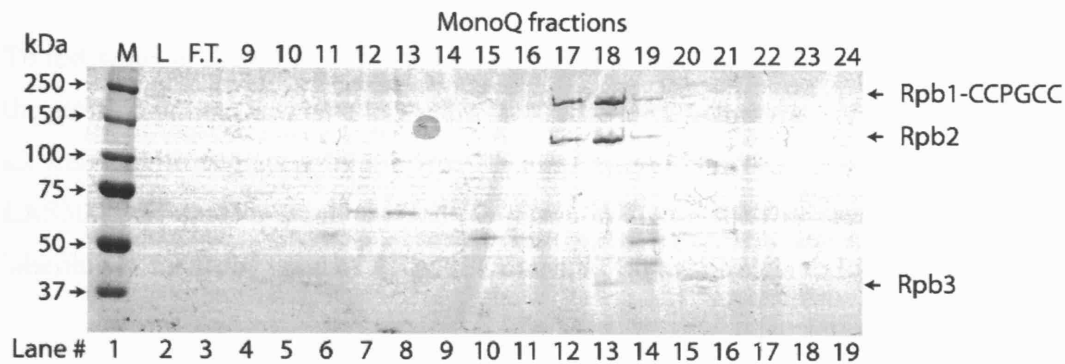
Panel A and B shows the same gel stained in different ways. Panel A is a Coomassie stained SDS-PAGE analysis of the purification of GST-CFP-CTD-CCPGCC on GST resin as described in Materials and Methods. Lysis of cells is very effective (compare lane 2 & 3 but the eluted protein is not very pure (see lane 7 & 8). Panel B shows the successful labelling of GST-CFP-CTD-CCPGCC following incubation with the Lumio green reagent, as per the manufacturer instructions. The picture was taken using a Las 3000 imaging system (Fuji).

### 4.9 Purification of Rpb1-HA-2Xa-CTD-myc-CCPGCC coreRNAPII

RNAPII incorporating a Rpb1-HA-2Xa-CTD-myc-CCPGCC (called RNAPII-CCPGCC from hereon) subunit was purified to near homogeneity using the procedure described in materials and methods, and protein from the last step of the procedure was analysed by 10% SDS-PAGE (see Figure 54).



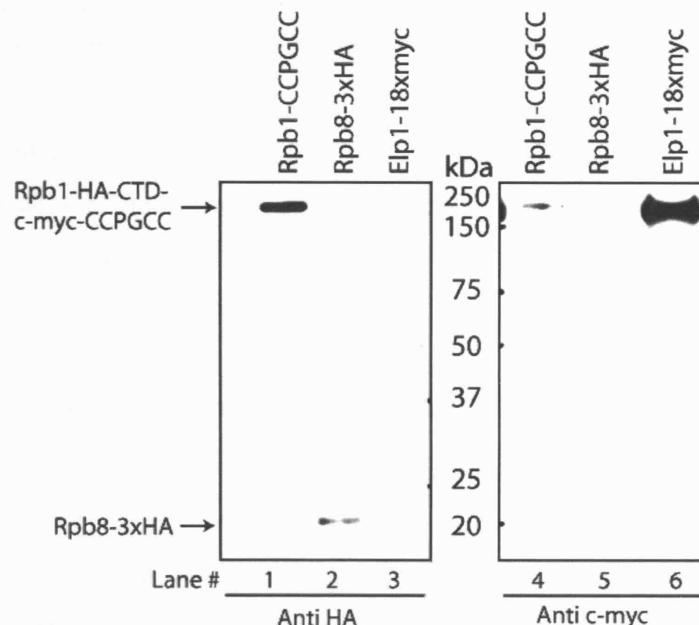
## Results Part II



**Figure 54 MonoQ elution profile of RNAPII-CCPGCC purification**

The MonoQ elution profile of RNAPII-CCPGCC. From each 1 ml fraction a 15  $\mu$ l sample was analysed by 10% SDS-PAGE and Coomassie stained. RNAPII-CCPGCC eluted in fractions 17-19 (lane 12-14). Only the 3 largest subunits of the RNAPII complex, are clearly visible in this gel, as indicated to the right.

To verify that the HA and c-myc tag was indeed present on the purified protein, an immunoblot using anti HA(12CA5) and anti c-myc (9e11) monoclonal antibodies was performed (see Figure 55). Purified Elp1-18myc (lane 3 & 6) was kindly provided by Catherine Greenwood as a positive control (for the presence of the myc tag). Purified Rpb8-3xHA RNAPII (lane 2 & 5) served as control for HA detection. Rpb1-CCPGCC (lane 1 and 4) shows clear signal from both the c-myc and HA epitopes on RNAPII.

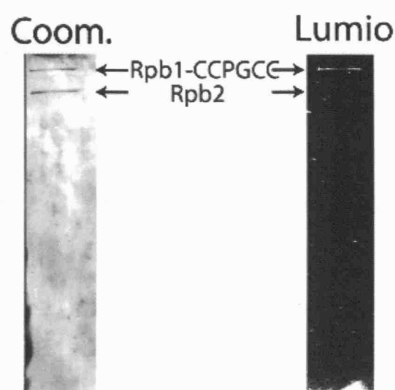


**Figure 55 Verification of presence of tags on RNAPII**

Immunoblot of purified RNAPII including Rpb1 tagged with HA and myc-CCPGCC (lanes 1 and 4). The membrane was cut and probed with anti-HA (12CA5) antibody (lanes 1-3) or anti-myc (9E11) antibody (lanes 4-6). Positive controls for the blot are purified RNAPII including the Rpb8-3HA tag (lane 2 and 6) and purified Elongator complex including Elp1-18 myc.



To test whether the CCPGCC-tag was amenable to labelling in the context of RNAPII, the purified protein was labelled before 10% SDS PAGE analysis, using a Lumio-green kit from Invitrogen according to manufacturer instruction. The gel was filmed using a LAS3000 system (Fuji) and the result is shown in Figure 56. This demonstrates specific labelling of the Rpb1 band of RNAPII (compare Coomassie stain to Lumio-stain)



**Figure 56 Labelling RNAPII with Lumio reagent**

*10% SDS-PAGE analysis stained by Coomassie and Lumio reagent. RNAPII with a CCPGCC tag at the C-terminal end was labelled using in-gel Lumio green detection kit (Invitrogen). The gel was filmed using a LAS-3000 light box (Fuji) to document the Lumio labelling (lane marked Lumio) and subsequently the same gel was Coomassie stained to reveal the proteins bands (lane marked Coom.). Only the two largest RNAPII subunits are visible because of poor Coomassie staining in this experiment. A fluorescent band only at the position of Rpb1 indicates that specific in-gel Lumio labelling of RNAPII was successful using the Lumio-kit and procedure. No significant labelling was observed when this kit was not used.*

Unfortunately, labelling of purified RNAPII-CCPGCC only worked when using Lumio-kit buffers (not shown) and the procedure specified in the manufacturer instructions. For reasons described in the paragraph below, this labelling strategy was not compatible with the experiments I planned to perform.

### **4.10 Labelling of the tetra-cysteine tag in solution**

Although labelling of the tetra-cysteine tagged proteins was possible, it was very inefficient. Labelling using the Lumio-Green kit (Invitrogen) only worked in the proprietary buffers. It was noted that the affinity of the FLaSH reagent for the tetra-cysteine tag depends on the cysteine residues being in the reduced state (Griffin et al., 2000). Full reduction ensures that the cysteine groups are free to react with the FLaSH reagent. This would normally be the case in the reducing environment of an *E. coli* cell

but *in vitro* such residues can be oxidized if proper precautions are not taken. Small quantities of reducing agents in buffers (such as 2-(N-Morpholino)EthaneSulfonic acid (MES) or Tris (2-Carboxy-Ethyl)-Phosphine-HCl (TCEP) are generally included for this purpose (Adams et al., 2002). TCEP has been reported to be a more stable reducing agent than DTT (Getz et al., 1999), and it is a more efficient reducer than  $\beta$ -mercaptoethanol, which also inhibits labelling at high concentration (Adams et al., 2002).

The Lumio green kit (Invitrogen) also contains a proprietary “enhancer” reagent. According to the manufacturer protocol, the proteins to be labelled are incubated at 70 °C for 10 min in the proprietary Lumio sample buffer containing Lumio green reagent before the enhancer is added. Denaturation of the tagged protein and reduction of the target cysteine groups probably are the aims of these steps. The enhancer chemical is not identified in the kit material, but its role is allegedly to limit unspecific labelling.

However, for my assays to work as planned, it was obviously necessary to avoid denaturing the protein in the labelling process. My project plan called for both a novel design of the CCPGCC context and an alternative procedure (using the Invitrogen FLAsH chemical) to label proteins in solution. Unfortunately, initial labelling experiments using the labelling buffer described in (Adams et al., 2002), but avoiding the heat denaturing step, failed to label significantly either RNAPII-CCPGCC or GST-CFP-CTD-CCPGCC. Thus, the procedure for labelling of native proteins in solution needed to be optimised.

### ***Tetra-cysteine labelled calmodulin for optimisation***

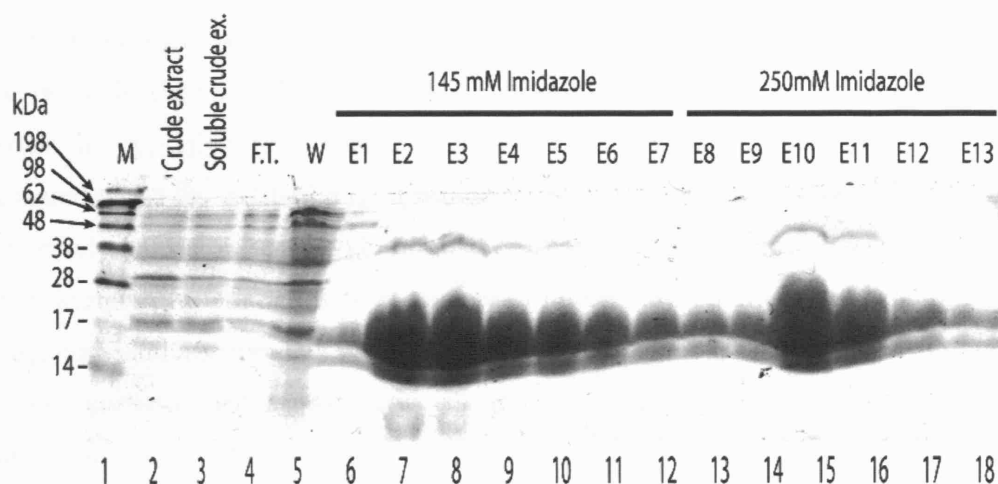
I wanted to optimise the labelling procedure using the Lumio reagent with exclusively known additives, in a non-denaturing buffer. The absolute amount of purified RNAPII and the concentration of the prep (~200  $\mu$ g/ml) precluded the use of this complex in optimisation experiments. In addition, as the sequence context of the CCPGCC-tag is identical in RNAPII-CCPGCC and GST-CFP-CTD-CCPGCC I expected results to be similar. To exclude the possibility that the context of the CCPGCC in my constructs was adversely affecting the efficiency of labelling in solution, I decided to optimise the labelling procedure on a protein carrying the published sequence context of the CCPGCC tag (Adams et al., 2002). Less efficient labelling due to a sub-optimal sequence context could mask any improvements during optimisation of the labelling procedure. Through the initial characterization of a protein, which had been used

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successfully by others before me for this purpose, I also hoped to show that the modified procedures, reagents and equipment employed were indeed functional.

A modified version of *X.laevis* calmodulin, where four residues in a N-terminal loop domain had been substituted for Cysteine (Cys6,7,10,11-calmodulin) was previously reported to be amenable to FIAsh labelling *in vitro* (Adams et al., 2002; Griffin et al., 2000). Calmodulin is a small (16.5 kDa) protein consisting of two lobular domains connected by a central  $\alpha$ -helix. Each of the lobular domains has two binding sites for  $\text{Ca}^{2+}$  ions (Chattopadhyaya et al., 1992). A construct for expression of Cys6,7,10,11-calmodulin in human cells (Adams et al., 2002), was obtained from the Tsien lab and the sequence was re-cloned into the bacterial gateway vector pET-DEST42 as described in Materials and Methods. Correct sequence was verified by sequencing and the plasmid was used to express Cys6,7,10,11-calmodulin in *E. coli* BL21(DE3) cells.

Using the C-terminal 6xHis tag, calmodulin was purified as described in Materials and Methods. The purification is shown in Figure 57. The protein expressed well and the protein was reasonably pure, but strangely appeared as a doublet, possibly caused by degradation or a contaminating *E. coli* protein.



**Figure 57 Purification and labelling of Cys6,7,10,11-calmodulin-6xHis**

Coomassie stained, 5-15% SDS-PAGE analysis of the purification of 6xHis-Cys6,7,10,11-calmodulin on NiNTA. F.T. = Flow through, W = Wash, E = Elution fraction. Column was eluted in batch with buffers containing two different imidazole concentrations as designated.

I decided that for the purpose of optimisation of Lumio labelling, the purity of this preparation would probably suffice, as specific labelling of single CCPGCC-tagged

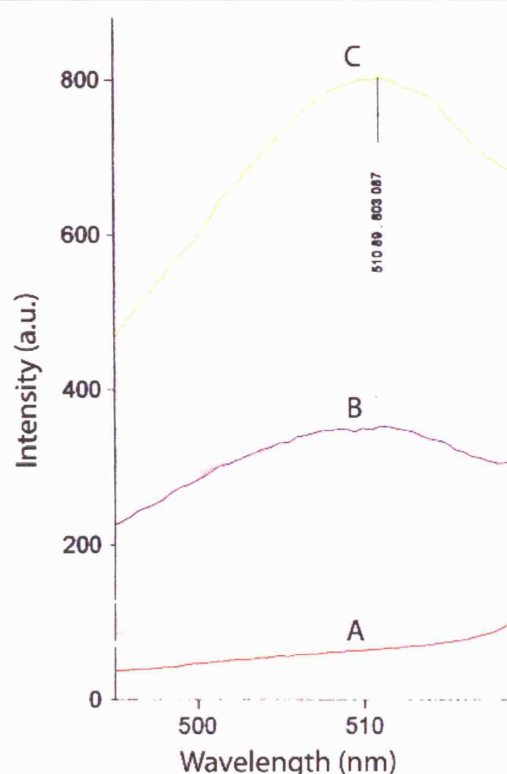
## Results Part II

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proteins in crude bacterial extract has previously been demonstrated (Adams et al., 2002).

To see if I could indeed label the tetra-cysteine tag in calmodulin I first tried to optimise the concentration of Lumio labelling reagent using the Lumio green kit buffers without the heating step. Lumio Green reagent concentrations of 1 $\mu$ M, 5 $\mu$ M and 10 $\mu$ M did not significantly label 1  $\mu$ M of Cys6,7,10,11-calmodulin-6xHis (not shown). Successful labelling was achieved at 20  $\mu$ M Lumio Green concentration, labelling 45  $\mu$ M of Cys6,7,10,11-calmodulin-6xHis (not shown). Thus, it seems that under these conditions the Cys6,7,10,11-calmodulin-6xHis protein could indeed be labelled efficiently in solution, while the GST-CFP-CTD-CCPGCC previously could not. At this point, I could not rule out that the context of the CCPGCC tag in the GST-CFP-CTD-CCPGCC protein adversely affects the efficiency of labelling. On the other hand, the concentrations of the proteins to be labelled were not the same, because GST-CFP-CTD-CCPGCC could not be concentrated to the level that Cys6,7,10,11-calmodulin-6xHis could without causing precipitation, which might also adversely affect labelling efficiency.

To further extend the line of proof-of-principle experiments with calmodulin, a Varian spectro-fluorometer was employed to detect the signal from the Lumio-green labelling. The result is shown in Figure 58. This apparatus operates in one of three principal modes: in excitation scan mode, the response at a known emission wavelength is measured while the excitation light source scans through a range of lower wavelengths. Conversely, emission scan mode holds the excitation light source at a set wavelength while scanning for emitted light at a range of higher wavelengths. In synchronous scan mode, both excitation and emission is measured simultaneously with a constant interval between emission and excitation wavelengths. The response is measured in arbitrary units of intensity. An excitation scan of Cys6,7,10,11-calmodulin-6xHis labelled in solution with Lumio green at room temperature is shown in Figure 58. Clear labelling of Cys6,7,10,11-calmodulin-6xHis is seen (compare curve A and B) but the effect after adding the enhancer is even clearer (compare curve B and C), giving a strong signal at the wavelength expected for Lumio Green.



**Figure 58 Fluorescence excitation scan with emission at 529 nm.**

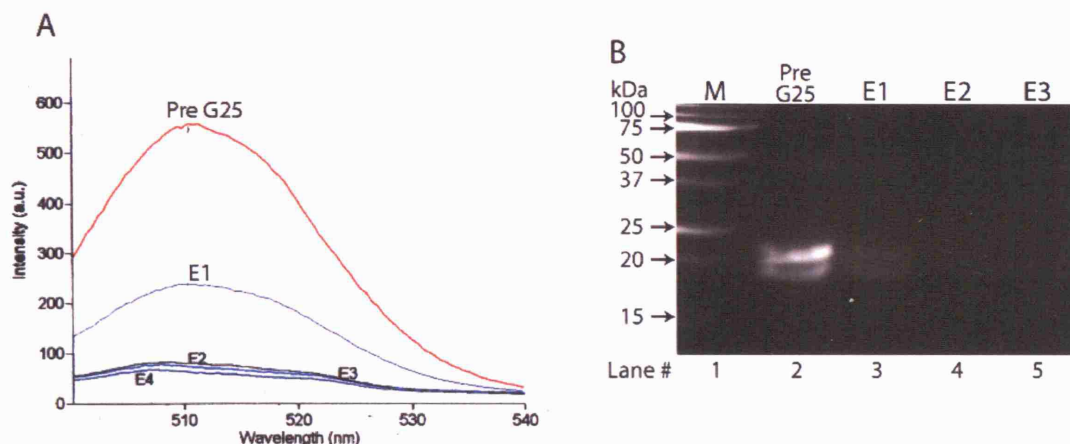
15  $\mu$ g Calmodulin tagged with the tetra cysteine tag was labelled by 1 hr incubation at RT with 20 $\mu$ M Lumio green reagent (curve B) followed by addition of 2  $\mu$ l Lumio green “Enhancer” for 6 hours (curve C). Curve A is a negative control without calmodulin containing Lumio green and enhancer. Fluorescence was measured in a Varian Eclipse spectro-fluorometer in a 386 well plate using excitation scan mode with emission set at 529 nm. The Lumio signal is significantly enhanced by addition of the proprietary “Enhancer” solution. The marker denotes the peak of intensity at 803 intensity units (arbitrary) at 510 nm as expected for the Lumio green reagent.

The FAsH reagent does not fluoresce unless both the sulphide groups are fixed through specific covalent coupling to the tetra-cysteine motif (Griffin et al., 1998) thus unspecifically bound FAsH molecules are lost to the reaction. Presumably, the enhancer inhibits unspecific binding thereby raising the effective concentration of reaction-competent FAsH giving rise to the stronger signal seen in curve C (figure Figure 58 ).

Together, the experiment described above demonstrated the feasibility of using the Invitrogen Lumio Green reagent as a labelling reagent in solution. But as the identity of the “Lumio green Enhancer” is undeclared in the manufacturer instruction, its effect on protein conformation cannot be appraised. The sample buffer used in these experiments might also contain reducing agents with unknown effect on protein conformation.

### ***4.11 Labelling of CCPGCC tag in solution***

The calmodulin peak fractions (Figure 57, lane 15-16) were pooled and dialysed into labelling buffer (see Materials and Methods) and labelled with Lumio green reagent as described in Materials and Methods. An initial incubation step in high concentrations of reducing agents was employed to fully reduce the cysteine residues in the CCPGCC tag. A number of different buffer formulations using high concentrations (10mM, 50mM, 100mM) of  $\beta$ -mercaptoethanol or DTT were tried. The buffers also contained low concentrations of MES which reportedly stimulates labelling (Adams et al., 2002). As high concentrations of DTT are inhibitory to labelling (Adams et al., 2002), the protein had to be diluted into labelling buffer after reduction before the labelling reaction, making it necessary to start with a concentrated protein solution. Thus Cys6,7,10,11-calmodulin-6xHis was concentrated to 1-3 mg/ml before labelling (see Materials and Methods). Gel-filtration, using G25 spin columns (Amersham) was used to remove the labelling reagents from the bulk protein before using the Varian spectro-fluorometer in excitation scanning mode to measure the emission at 528 nm (Lumio Green emission peak), while scanning across wavelengths appropriate for Lumio Green excitation (508 nm). Successful labelling was achieved using 20  $\mu$ M Lumio Green reagent in [50mM MOPS pH=7.4, 2 mM MES, 1mM TCEP, 1mM EDTA] at RT for 4 hours. The result is shown in Figure 59, panel A. The protein concentration in the labelling reaction was 0.25 mg/ml. Significant absorption around 508 nm is evident, indicating that labelling had been successful. The signal drops sharply after G25 purification (compare curve "Pre G25" with curve "E1") making it hard to judge whether the signal is specific to the labelled protein. To further confirm labelling, the protein was analysed by 15% SDS-PAGE and the result is shown in Figure 59 panel B. The labelling shows that the drop in signal correlates well with the concentration of protein indicating that the signal indeed is specific. The reason for the loss of protein upon G25 gel filtration is unknown. As the cut-off value for the Sephadex G25 resin is around 5 kDa (according to manufacturer instruction) and as calmodulin is 16.5 kDa it should partition exclusively to the void volume. However, the protein might stick to G25, possibly through hydrophobic interaction. Despite the protein migrating as a doublet (suggesting degradation or contamination), the proteins in both bands clearly retain the N-terminal of Cys6,7,10,11-calmodulin-6xHis.



**Figure 59 Labelling of calmodulin using Lumio Green**

*Cys6,7,10,11-calmodulin-6xHis* was labelled o.n. and the labelling buffer was exchanged for PBS using G25 spin columns (Amersham). Panel A shows the result of an excitation scan of the G25 elutions using a Varian Eclipse 3000 fluorometer as described in Materials and Methods. Emission wavelength was set at 528 nm. Panel B shows a 15% SDS-PAGE analysis of the same samples. Lane 2 shows the G25 input and lanes 3-5 show the elutions from the G25 spin column. Although, *Cys6,7,10,11-calmodulin-6xHis* seems to stick non-specifically to the G25 column, specific labelling of calmodulin is demonstrated. Panel B picture recorded using a LAS-3000 (Fujii) gel documentation system.

The experiment above shows that the Lumio green reagent can indeed be used to label native conformation proteins in solution if they carry the tetra-cysteine tag in the context of *Cys6,7,10,11-calmodulin*. Unfortunately, the GST-CFP-CTD-CCPGCC was not amenable to efficient labelling by the same procedure. One problem could be that the starting protein concentration was much lower than that of *Cys6,7,10,11-calmodulin-6xHis*. As mentioned above, attempts to concentrate GST-CFP-CTD-CCPGCC further unfortunately resulted in protein precipitation. Different preparations of GST-CFP-CTD-CCPGCC had different degrees of internal CFP fluorescence and problems with solubility of the fusion protein upon harvest were also encountered (not shown). These problems may all relate to a problem of CFP-folding. Fluorescent proteins fused to flexible domains (like the CTD) have indeed been reported to fold much less efficiently than they would on their own (Cabantous et al., 2005; Waldo et al., 1999) and ill-formed proteins are prone to precipitation. Alternatively, the context of the CCPGCC tag in the GST-CFP-CTD-CCPGCC protein may render the labelling process inefficient because the FLAsH chemical can not interact successfully with the tetra cysteine tag. The problem may obviously also be compounded by a combination of these factors.



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In an attempt to increase CFP-CTD-CCPGCC solubility (in order to be able to prepare the protein at a higher concentration), the N-terminal tag was changed from GST- to 6-His using the Gateway destination vector pDEST-17. The cloning is described in Materials and methods and the purification is shown in Figure 60.



**Figure 60 Purification of 10xHis-CFP-CTD-CCPGCC**

Coomassie stained 10% SDS-PAGE analysis of 10xHis-CFP-CTD-CCPGCC purification. The protein is fairly pure though there appears to still be a problem with solubility (compare lane 2 and 3) shown by the preferential precipitation of the fusion protein upon clearing of the crude extract by centrifugation.

The purification procedure involved washing the column with very high concentrations of imidazole (see lane 7 -14) in order to disrupt unspecific binding of the CTD fusion protein and contaminants to the NiNTA resin. This behaviour of a CTD fusion protein has been reported previously (Morris et al., 1997). Because of time constraints, the new protein has not yet been concentrated and tested with the newly established *in vitro* labelling procedure.

### 4.12 Control for measuring FRET between the N- and C-term of calmodulin

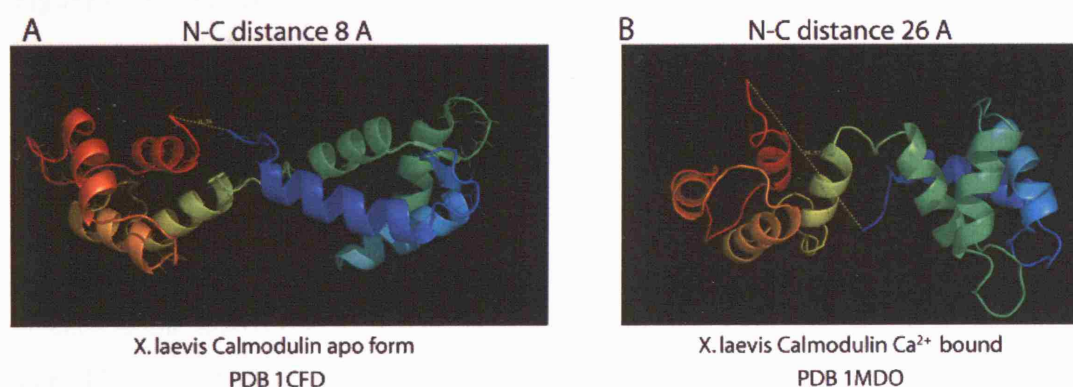
As a positive control for FRET between the CCPGCC tag on the N-terminal of Cys6,7,10,11-calmodulin and the fluorescent protein, a construct which placed the two in close proximity to each other was needed. The solution NMR structure of *X laevis* calmodulin (PDB: 1CFD) showed that the distance from C- to N-terminal is 13 Å in its  $\text{Ca}^{2+}$  free, apo-form. In a Calmodulin-CFP fusion protein, the distance from the CFP surface to its fluorescent centre (10 – 15 Å (Hoffmann et al., 2005)) would have to be added, giving a total distance of 28 Å between fluorophores. This is less than the Förster distance for the CFP/YFP pair in a fusion protein (37 Å) (Tsien, 1998). Consequently, I



## Results Part II

decided to fuse CFP to the C-terminal of 6xHis-Cys6,7,10,11-calmodulin. The details of the construction are described in Materials and Methods.

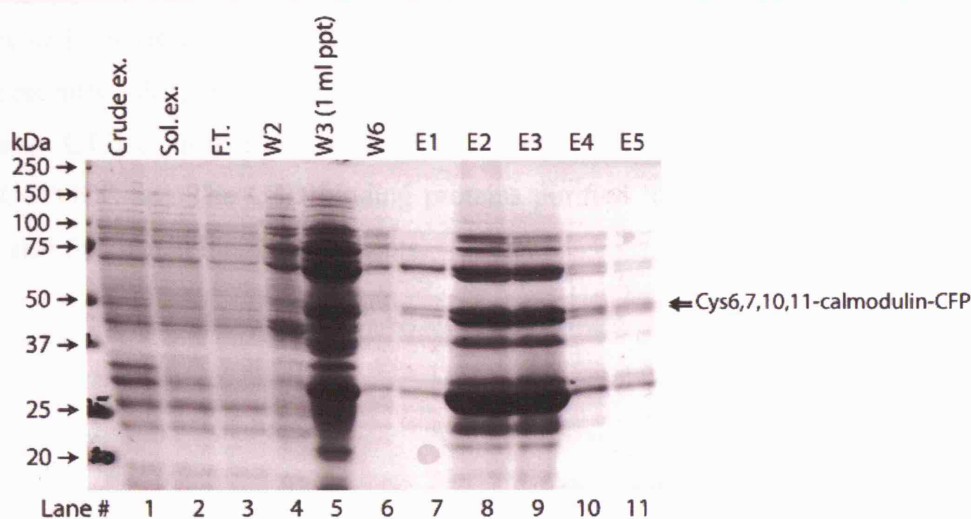
Because calmodulin extends in the presence of calcium ions, another advantage of such a fusion protein would be the ability to test if changes in distance between FAsH and CFP can actually be measured in real time by FRET, as would be required for the RNAPII CTD experiments. Compared to the apo-form, the solution structure of *X. laevis* calmodulin bound to  $\text{Ca}^{2+}$  shows that the distance between N- and C- terminals is extended by 18 Å to 26 Å (Zhang et al., 1995). The extension is reportedly caused by calcium-induced rigidity of the central helix (Hertadi and Ikai, 2002; Ishida et al., 2000). The two different calmodulin conformations are illustrated in Figure 61.



**Figure 61 Calmodulin conformational change upon  $\text{Ca}^{2+}$  binding**

*Illustration of the difference in distance between N- and C- terminals of *X. laevis* calmodulin in the absence (panel A) or presence (panel B) of  $\text{Ca}^{2+}$ . The proteins are coloured from blue N-terminals to red C-terminals. Figure made using the program Pymol*

The protein was expressed in *e coli* cells and purified as described in Materials and Methods. The result of the purification is shown in Figure 62. High Imidazole concentration elutes a number of bands from the column (lane 7-11) but only the doublet band (indicated with arrows) is absent from the wash fraction (lane 6) suggesting specific elution of the fusion protein. Higher concentrations of Imidazole in the binding and washing steps may be necessary to obtain purity. Due to lack of time, the fraction was not been tested with Lumio in-gel staining to detect bands arising from tagged protein.



**Figure 62 Cys6,7,10,11-calmodulin-CFP purification**

Coomassie stained 10% SDS-PAGE analysis of purification. 5  $\mu$ l of each elution fraction (E) was loaded per lane. The proteins in lane 5 were concentrated from 1 ml using Strataclean resin (Stratagene). The elution peak contains many contaminating bands but the annotated bands are specifically and quantitatively depleted from the soluble extract by the column, indicating that they stem from Cys6,7,10,11-calmodulin-CFP

A very high purity of the Cys6,7,10,11-calmodulin-CFP protein may not be of the essence as the interaction to be probed is intramolecular and certainly the FRET signal should be measurable, even in a rather crude preparation like this.

#### 4.13 Summary

In conclusion, a number of proteins (GST-CTD, Fcp1, Ess1 and Rsp5) have been purified with the view of investigating their effect on RNAPII-Mediator interaction using Biacore. Preliminary experiments have been undertaken to determine the conditions under which the assay can be performed.

Furthermore, a system for FRET detection of CTD conformational change has been designed and constructed. A labelling procedure has been developed using the commercially available Lumio Green reagent (Invitrogen) to label folded proteins in solution. Results indicate that the context of the tetra-cysteine tag has a large influence on the efficiency of labelling *in vitro*. With further buffer optimisation, this hurdle may be overcome. Alternatively, the CFP-CTD-CCPGCC constructs will be re-cloned to put the CCPGCC tag in the Ac-WDCCPGCCK-NH<sub>2</sub> context (Adams et al., 2002).

A control construct for detecting FRET between CFP and the FLaSH fluorophore has been designed and purified.

## Results Part II: Summary

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As regards the RNAPII-CCPGCC protein, ongoing efforts are now focused on using fluorescently labelled antibodies against the epitope tags at either end of the CTD to measure CTD conformation, pending development of a suitable labelling protocol for the CCPGCC tag. The CTD binding proteins purified for use in Biacore experiments will also find use in these experiments.

### **5 Discussion**

The interaction of the Mediator complex with RNAPII, and the mechanism and result of RNAPII CTD phosphorylation have been the major focus of the work underlying this thesis. The questions pursued in this thesis were raised by observations from fractionation of yeast extracts into a soluble, chromatin-free protein fraction and an insoluble chromatin-bound protein fraction. These studies demonstrated that Mediator is almost entirely absent from the chromatin bound RNAPII engaged in transcription, while it is present in the soluble fraction where only RNAPIIA is detected (Svejstrup et al., 1997). Other experiments showed that Mediator could dynamically exchange from one RNAPII to another. Taken together, this prompted a model of cyclic association and disassociation of RNAPII and Mediator, depending on CTD phosphorylation status, i.e. that CTD phosphorylation might disrupt the Mediator-RNAPII interaction, releasing RNAPII from promoters. However, preliminary experiments had failed to prove or disprove the model, and the possibility that additional events other than CTD hyperphosphorylation were needed to dissociate Mediator and RNAPII was raised (Svejstrup et al., 1997).

The main results from my experiments and their implications will be discussed in the following.

#### **5.1 Immobilisation can inhibit hyperphosphorylation of RNAPII**

Considerable effort was invested in establishing an experimental system enabling me to address whether Mediator-RNAPII interaction could be disrupted by CTD phosphorylation. Unfortunately, the initial system had to be abandoned, because immobilization of RNAPII via the tag on Rpb2 severely inhibited CTD phosphorylation. The reason for the observed inhibition is unknown. In and of itself, the Rpb2-HisHA tag did not cause any detectable phenotypes in yeast, indicating that RNAPII/Rpb2-HisHa is fully functional *in vivo*. Even after optimisation of buffer conditions, quantitative CTD phosphorylation did not occur. Interestingly, no intermediate bands between the -IIA band and -IIO Rpb1 band were observed, which might suggest that in a small subpopulation of immobilised RNAPII, processive phosphorylation was indeed taking place. In any case, the problem was solved by moving the RNAPII tag to Rpb8 (which is on the opposite side of the polymerase relative to the CTD), suggesting that steric hindrance of kinase access to the CTD was

## Discussion

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indeed part of the problem. The inhibition fits well with the concept of a compact random coil structure of the CTD, which would presumably be more easily obstructed than an extended conformation that might escape obstruction. Phosphorylation of Rpb2-tagged immobilized RNAPII using the much smaller TFIIF or CTDK-1 complexes was not attempted, but could potentially be used to address the basis for the inhibition. If steric clashes are indeed the whole explanation, it might suggest that TFIIF needs to have access to the base of the CTD, so that its phosphorylation of the CTD repeats cannot occur as efficiently if this access is perturbed. A case can be made for a preference of Kin28 to start processive phosphorylation at the base of the CTD. As mentioned in the introduction, Kin28 has been shown to interact with Rpb1 and Rpb2 by far western (Feaver et al., 1994). Moreover, the Ser5→E substitution of the five most N-terminal repeats in the C-terminal domain was reported to be lethal (West and Corden, 1995). The negatively charged glutamate side chain is supposed to mimic a constitutively phosphorylated serine, and Kin28 may conceivably have less affinity for a repeat already phosphorylated on Ser5. Thus if the Ser5→E substitution indeed results in a general problem for initiation of processive Kin28 phosphorylation of the remaining CTD repeats, this might be the reason for the lethality observed and also be the reason that I failed to efficiently hyperphosphorylate RNAPII, immobilised via the Rpb2 subunit.

Other mechanisms for inhibition might also be envisaged. For example, the association of an 8WG16 antibody with the CTD of stalled *D. melanogaster* RNAPII can trigger its release from the DNA template in vitro (Zhang et al., 2004), indicating that factors interacting with the CTD can somehow affect the general conformation of the polymerase complex. Inhibitory effects due to immobilisation by the HisHA tag *per se* that are relayed from Rpb2-HisHA through the RNAPII complex to inhibit the proper association of TFIIF with RNAPII can thus not be ruled out.

### **5.2 Phosphorylation of the CTD by TFIIF, dissociates holoRNAPII in vitro**

The experimental work described above leads to the conclusion that hyperphosphorylation of the RNAPII CTD is able to effect considerable dissociation of RNAPII and Mediator *in vitro*.

The early observation that RNAPII enters the PIC in the IIA form and leaves it in the IIO form prompted the notion that the initiation to elongation transition might be

## Discussion

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facilitated by hyperphosphorylation of the CTD (Payne et al., 1989). As the transcription elongation complex nucleates around RNAPII, and transcription progresses into the elongation phase, the Mediator complex stays behind on the promoter and thus connections between the two complexes must be severed. The present work provides direct biochemical evidence supporting the notion that phosphorylation helps to break the connection. This finding is novel and has not previously been shown in the context of holoRNAPII.

In the yeast system, Mediator contacts the CTD of RNAPII, but also has interactions with domains outside of the CTD. Notably, parts of the Mediator middle region contacts Rpb1, Rpb11 and Rpb6, while other parts interact with Rpb3 and Rpb11. Via its “flap regions” the Mediator Head domain interact with areas on Rpb1 and Rpb2 (Davis et al., 2002). Unless invoking conformation changes in RNAPII or Mediator that bring about the separation, the implication of my results is thus that the CTD is the primary means of interaction between the two complexes in the holoenzyme.

How does this relate to the pre-initiation complex? The theory of general recruitment of the holoenzyme to promoters (Kim et al., 1994); Thompson, 1995 1201 /id} has been contested by ChIP experiments showing Mediator arriving minutes before RNAPII (Bryant and Ptashne, 2003; Cosma, 2002). As Mediator and RNAPII thus may be recruited sequentially, whatever contacts exist between them in the PIC must probably be established during PIC formation. Modelling of the PIC based on several lines of evidence ((Chen et al., 2004; Juo et al., 2003; Tsai and Sigler, 2000) suggested that TFIIB, TBP and DNA are located in the interface between RNAPII and Mediator (Asturias, 2004). The effect of these subunits on the stability of the Mediator-RNAPII is unknown, but if they serve to destabilise the connection, this might make Mediator-RNAPII interaction through the CTD domain more important.

In experimental support of this notion, an investigation of the stability of PIC's assembled in crude yeast nuclear extract found that phosphorylation of RNAPII (either by TFIIF or Cdk8/CycC/Med12/Med13) caused ATP-dependent dissociation of the PIC (Liu et al., 2004). However, previously unknown targets for the two kinases were also identified in that study and whether dissociation was a direct or indirect consequence of CTD phosphorylation could in any case not be distinguished. The data showed that TFIIF has two targets in Mediator (Med4 & Med14) and two others were TFIID components (Bdf1 and TAF2). Given the multiple subunit interactions in the PIC there was no clear conclusion on the functional effect of these phosphorylation events. Later,

point-mutation of one of the sites in Mediator (Med4, Thr-237→Ala) was shown to have no significant effect on viability (Guidi et al., 2004), suggesting that this phosphorylation event does not serve a crucial function.

The finding that CTDK-1 can dissociate the holoRNAPII complex on its own (see below) certainly suggests that the non-CTD targets of TFIIH/K are not important for the RNAPII-Mediator dissociation observed here, as one would not expect CTDK-1 to have the same targets as TFIIH. Indeed, I failed to observe phosphorylation of Mediator subunits by CTDK-1 (data not shown).

### ***5.3 Dissociation of holoRNAPII is not dependent on holoTFIIH in vitro***

The CTD kinase TFIK is able to effect dissociation of holoRNAPII outside of the holoTFIIH context.

The coreTFIIH subunit Tfb1 has been reported to interact physically with the Mediator Gal11 subunit *in vitro* (Sakurai and Fukasawa, 1998) and the TFIK subunit Kin28 was shown by far-western to interact with Rpb1 and Rpb2 (Feaver et al., 1994). Thus, a priori one might assume that the dissociation mechanism of holoRNAPII is dependent on a combination of protein-protein interaction and CTD phosphorylation. As ATP-dependent holoRNAPII dissociation can be caused by TFIK kinase, this leads to the conclusion that all coreTFIIH subunits are dispensable for the dissociation mechanism per se. The question that followed was whether a combination of Kin28-RNAPII contacts and CTD hyperphosphorylation were needed for dissociation to take place and whether dissociation was dependent on the site of phosphorylation.

### ***5.4 Phosphorylation of the CTD by CTDK-1 dissociates holoRNAPII in vitro***

The Ser2 specific CTD kinase CTDK-1 can indeed bring about Mediator-RNAPII dissociation and so it can be concluded that phosphorylation per se is sufficient for holoRNAPII dissociation.

The dissociation of holoRNAPII upon phosphorylation of Ser2 is perhaps surprising because holoRNAPII dissociation *in vivo*, probably at the promoter clearance stage, is associated primarily with Ser5 hyperphosphorylation (Jiang et al., 1996), whereas Ser2P phosphorylation is only observed later in the transcription cycle (Cho et al., 2001). The simplest explanation for the ability of CTDK-1-mediated phosphorylation to disrupt RNAPII-Mediator interaction is that charge repulsion, rather

## Discussion

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than specific alteration in CTD tertiary structure, might underlie the observed dissociation. As mentioned in the introduction, direct interaction of human Mediator complexes with acidic activator domains, can elicit Mediator conformational change (Taatzjes et al., 2002) and furthermore, in the human system, Mediator adopts a specific CTD-dependent conformation (Naar et al., 2002). The negatively charged hyperphosphorylated CTD may thus serve to interact with and change Mediator conformation in the holoRNAPII complex to affect RNAPII release.

Although CTDK-1 is the main Ser2 kinase *in vivo* (Cho et al., 2001), it was reported that CTDK-1 is also able to phosphorylate some Ser5 positions, as well as the Ser2 positions, of the yeast CTD fused to GST *in vitro* (Jones et al., 2004). Figure 35 shows a super-shift elicited by CTDK-1 phosphorylation of RNAPII, which was pre-phosphorylated on Ser5 by saturating amounts of TFIIK. The super-shift implies that saturating phosphorylation by the specific kinase TFIIK still leaves the CTD with many potential targets for CTDK-1. Given the Ser5/Ser2 specificity of CTDK-1 shown against CTD peptides (Jones et al., 2004) this suggests that Ser2 is targeted by the CTDK-1 kinase purified here. Interestingly, in our hands, CTDK-1 does not seem to phosphorylate Ser5 to any great extent. This was implied by experiments done by Somesh Baggavalli (Svejstrup lab) in which TFIIK and CTDK-1 kinases (purified by the protocol described here, and used under identical conditions) were used separately to create RNAPIIO (Somesh et al., 2005). The Ser5 specific phosphatase Ssu72 was then used to dephosphorylate RNAPIIO. Only the TFIIK- phosphorylated RNAPII changed position to a higher mobility, whereas the CTDK-1-phosphorylated RNAPIIO was unchanged. This indicates that only a few, if any, Ser5P residues are present in the CTDK-1 hyperphosphorylated CTD. The kinetics of CTDK-1 activity was reported to depend critically on reaction conditions (Morris et al., 1997). Differences in the CTDK-1 purification procedure, the assay conditions, or the substrate may explain the discrepancy between my results and those of (Jones et al., 2004).

In summary, the above experiments have shown that yeast holoRNAPII can be dissociated into Mediator and RNAPII by hyperphosphorylation of the CTD *in vitro*, and that this dissociation requires no additional factors. This answers the question that was posed at the outset of this investigation.



## Discussion

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The purified RNAPII-Mediator complex ((Kim et al., 1994); this thesis) contains only unphosphorylated RNAPII, and a preference for binding the RNAPIIA form was also previously reported for a mammalian protein complex, NAT, containing Mediator orthologs (Sun et al., 1998). Co-precipitation of RNAPIIB and RNAPIIA, but not RNAPIIO, thus indicated that NAT preferentially interacts with RNAPIIA *in vitro*. These observations support the idea that Mediator has a lower affinity for the phosphorylated form of RNAPII than the un-phosphorylated, in agreement with the data on phosphorylation-mediated disruption presented here. The interaction of RNAPIIA and Mediator is dynamic, as shown by the ability of Mediator to disassociate from holoRNAPII and re-associate with coreRNAPII bound on beads (Svejstrup et al., 1997). The present study of the interaction of Mediator with RNAPIIO relies on end-point assays. Using Biacore it might be possible to measure association and disassociation in real time, and possibly this would reveal whether phosphorylation of RNAPII simply changes the affinity of Mediator for RNAPII, or whether structural changes of Mediator or RNAPII might also be involved. Preliminary Biacore experiments were conducted where holoRNAPII was captured, via a 12CA5 antibody, on a Biacore CM5 chip covered with anti-mouse IgG. The idea was to phosphorylate holoRNAPII on-chip by adding kinase with or without ATP, and then to monitor the dissociation of holoRNAPII in real time. Regrettably, my preliminary findings showed an unexpectedly low signal from the bound holoRNAPII relative to the molecular mass of the bound capturing antibody. The signal could be out-competed by including 1 mg/ml HA peptide, indicating that the signal obtained was specific. Presumably, the concentration of purified holoRNAPII competent to bind the chip was simply too low. Purification in much larger scale to obtain more holoRNAPII protein will be necessary to pursue this line of experiments, especially in the case where Mediator is immobilised, and RNAPIIA or -IIO is passed over the chip.

In the absence of real time data on the dissociation of holoRNAPII, the detailed mechanism of dissociation remains unknown.

In the course of the work on Mediator-RNAPII dissociation, the functional interaction between Mediator and the CTD kinases has been studied and some novel observations have been made. These will be discussed below.

### ***5.5 HoloTFIIH is dispensable for Kin28 stimulation by Mediator***

Mediator stimulates the CTD kinase activity of the TFIID complex *in vitro*.

It was known that Mediator strongly stimulates the CTD kinase activity of holoTFIIH (Kim et al., 1994; Myers et al., 1998). Here, it is shown that the context of holoTFIIH is dispensable for Mediator stimulation of TFIID. The finding that Mediator can stimulate TFIID activity *in vitro* had not been reported by others when I observed it, but was later published by the Myers lab (Guidi et al., 2004). In that paper, Mediator was shown to stimulate phosphorylation of GST-CTD as well of RNAPII, suggesting that TFIID interaction with the non-CTD RNAPII complex is not actually absolutely required for stimulation. Indeed, it could be surmised that binding of the CTD peptide by Mediator complex is enough to “present” a more accessible target to kinases. For example, the free CTD peptide could maintain some secondary structure, which is inhibitory to processive phosphorylation by TFIID, and Mediator interaction might disrupt this secondary structure allowing TFIID to phosphorylate the CTD processively.

In seeming contrast to the observations of Myers and colleagues stand observations in the *S. pombe* system, where TFIID kinase activity towards *S. pombe* coreRNAPII or holoRNAPII was compared *in vitro*. In this system, only *S. pombe* TFIID was stimulated by the presence of Mediator, while *S. cerevisiae* TFIID was not (Spahr et al., 2000), suggesting that, given the conservation of the CTD sequence, *S. pombe*-specific contacts outside of the CTD influence stimulation of CTD phosphorylation. Alternatively, stimulation also entails Mediator-TFIID interaction, which might be species-specific.

### ***5.6 CTDK-1 is stimulated by Mediator in vitro***

Purified Mediator stimulates the kinase activity of CTDK-1 *in vitro*. Such stimulation has not been reported previously. In the course of investigating the stimulation of TFIID by Mediator, the Myers lab also tried, but failed, to see stimulation of the Cdk8/CycC/Med12/Med13 kinase complex by Mediator *in vitro*. As the activity of the Cdk8/CycC/Med12/Med13 complex is characterized by distributive kinetics, while both TFIID and CTDK-1 are processive CTD kinases, they may conceivably differ as to their substrate preferences and basic mechanism. As no physical interaction between CTDK-1 and Mediator has been reported, the stimulatory effect of Mediator may exclusively be caused by favourable “presentation” of the substrate to the kinase. To rule out any

effects caused by the non-CTD parts of the RNAPII complex, it will be interesting to investigate in the future whether CTDK-1-mediated phosphorylation of a GST-CTD fusion protein can also be stimulated by Mediator.

### ***5.7 CTDK-1 kinase is stimulated by Rsp5-GST***

The observation that Rsp5-GST can stimulate CTDK-1 activity is novel and intriguing, and may suggest that the basis for stimulation of CTD-phosphorylation is not intrinsic to the Mediator complex or the kinase, but rather a consequence of a change in CTD-presentation elicited by the binding of a protein. Based on this result it is thus tempting to speculate that the flexible, free CTD in some way limits progressive phosphorylation, and that this inhibition is relieved by the binding of (at least certain) protein partners. It would be interesting in the future to repeat the Rsp5 experiment using GST-CTD to see if the non-CTD part of RNAPII participates in the stimulation mechanism. Interestingly, the recent finding that Rsp5 does not bind CTD-less RNAPII (B. Somesh, personal communication) could be viewed as an argument against an involvement of the rest of the polymerase. Alternatively, an interaction between Rsp5 and CTDK-1 could be invoked to explain the observed stimulation of CTD phosphorylation. This possibility could also be pursued with the reagents already at hand. At this point, I favour the idea that the binding of certain proteins to the CTD fixes it in a conformation, which is amenable to efficient phosphorylation.

Rsp5 has been shown to be the E3 ubiquitin ligase for RNAPII stalled in a ternary complex (Somesh et al., 2005). The ubiquitylation leads to the degradation of stalled RNAPII *in vivo*, ostensibly serving to remove such blocks to transcription of potentially essential genes (Svejstrup, 2003). Ubiquitylation of free RNAPII is inhibited to different degrees by either Ser2 or Ser5 phosphorylation but incorporation of RNAPII into a ternary complex with DNA and RNA specifically relieves the inhibition caused by Ser2P, but does not significantly affect the inhibition caused by Ser5P (Somesh et al., 2005). Thus, in this *in vitro* system, Rsp5 stimulation of CTDK-1 should not a priori, affect ubiquitylation of stalled RNAPII which would fit well with its role as a E3 ubiquitin ligase. Future *in vitro* work may establish if this is indeed the case. Indeed, DNA damage in *S. cerevisiae* was reported to correlate with an increase in global RNAPII Ser2 phosphorylation (Ostapenko and Solomon, 2003), possibly linking increased CTDK-1 activity with recruitment of Rsp5 to stalled RNAPII. However, the

physiological role (if any) of the Rsp5 stimulation of CTDK-1, remains entirely to be determined.

The idea that protein-binding might fundamentally alter the structure of the CTD forms the basis for the experiments described in the second part of the thesis. The experimental system I began establishing for this purpose, as well as the results that might be expected by its use, are briefly discussed below.

### ***5.8 Measuring CTD conformation by FRET***

A system was developed to study responses in CTD conformation to modification, or occupation by CTD-interacting factors. The ambition is to help elucidate a long standing conundrum: Phosphorylation-dependent changes in the space occupied by the CTD in solution have been described in several papers (Laybourn and Dahmus, 1989; Zhang and Corden, 1991b), and in 2D crystal structures of RNAPII, the CTD was reported to occupy a space 4 times its theoretical volume (Meredith et al., 1996) corresponding to a random coil structure (Meinhart and Cramer, 2004). On the other hand NMR analysis of short CTD peptides (Harding, 1992; Kumaki et al., 2001; Noble et al., 2005) or indeed a 8 repeat CTD peptide (Cagas and Corden, 1995) failed to find evidence of a rigid structure. In addition, no significant change in conformation was detected upon phosphorylation (either Ser2 or Ser5) of a three-repeat sequence (Noble et al., 2005). Structural analysis of the interaction of CTD peptides with different CTD interacting domains have been reported to elicit different repeat conformations ranging from an extended coil-type structure (Verdecia et al., 2000) to a non-extended  $\beta$ -fold structure (Fabrega et al., 2003). NMR analyses of conformational dynamics of the whole CTD with a CTD interacting protein have not been reported, perhaps due to technical difficulties in synthesising long CTD peptides with well-defined phosphorylation patterns (Jones et al., 2004).

The FRET-based system described may be able to measure, in real time, the overall effects of phosphorylation or indeed isomerisation of the full-length CTD, and also the effect of CTD binding proteins. If various CTD binding proteins give rise to differences in CTD expansion, this may offer clues as to their individual mode of binding as well.

## **6 Future experiments**

### **6.1 Ternary complex effects on dissociation**

The possibility that CTD phosphorylation (or indeed association with CTD kinases) can cause conformational changes in RNAPII has been mentioned previously (Liu et al., 2004; Sun et al., 1998). The presence of holoRNAPII in a ternary complex with a DNA:RNA hybrid and the non-template strand may change the interaction with Mediator by changing RNAPII conformation. As mentioned above, this possibility is currently under investigation by assembling holoRNAPII onto an immobilised DNA/RNA hybrid as described (Kireeva et al., 2000; Somesh et al., 2005), before performing a dissociation assay. Preliminary results suggest that indeed some Mediator is displaced by the addition of the non-template strand to a holoRNAPII/RNA/DNA template strand complex (data not shown).

### **6.2 Elongator effects on dissociation**

The Elongator complex was proposed to compete with the Mediator complex for binding the CTD at the initiation-elongation transition (Otero et al., 1999). The hypothesis was based on the observation that Elongator preferentially interacts with the phosphorylated form of RNAPII during fractionation (Otero et al., 1999), and that Mediator is absent from fractions containing the elongating form of RNAPII (Svejstrup et al., 1997). However, the dissociation experiments described here indicate that Elongator is in fact not necessary for holoRNAPII dissociation to occur. Recently, RNA chromatin immunoprecipitation of Elongator subunits shows that the complex is tightly associated with nascent pre-mRNA throughout the coding sequence in yeast genes (Gilbert et al., 2004), so possibly Elongator could play a role in holoRNAPII conformation by binding RNA, analogous to the way the Pcf11 protein has been reported to be able to dislodge RNAPII from the chromatin template by binding the CTD and RNA at the same time (Zhang et al., 2005b). I have undertaken preliminary experiments using controlled transcription on immobilised templates to create stalled transcription complexes with accessible nascent mRNA with the view of enabling an investigation of the effects of adding purified Elongator in this system in the future (data not shown).

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